Targeted induction of apoptosis for cancer therapy

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chapter 1

Introduction to the thesis

Introduction to the thesis

Programmed cell death, known as apoptosis, is an essential cellular homeostasis mechanism that ensures correct development and function of multi-cellular organisms. The pivotal importance of the correct execution of apoptosis is apparent from the many human diseases with aberrancies in apoptosis, including cancer. During cancer development, various imbalances can arise in the apoptotic machinery. Consequently, sensitivity towards apoptosis is progressively reduced, which ultimately leads to inappropriate cell survival and malignant progression. However, it has become clear that cancer cells are often reliant on these aberrancies for continued survival. Perhaps counter intuitively, cancer cells can in fact be more prone to apoptosis than normal cells. The apoptosis-prone phenotype of cancer cells is masked and counterbalanced by upregulation of one or more anti-apoptotic mechanisms. Therefore, it is of enormous therapeutic interest to selectively tip the balance of the cellular fate of cancer cells towards apoptosis.
Indeed, the rational design of novel agents that can selectively induce apoptosis in cancer cells is a rapidly developing field, as exemplified by the plethora of such agents reported in contemporary literature. In **chapter 2**, a selection of novel rationally designed anti-cancer approaches that target apoptosis-related aberrancies is discussed. The aim of this chapter is to integrate new insights in apoptosis biology with recent progress in the different fields of targeted strategies. This integration of concepts might add to the development of a new therapeutic approach perhaps best described as "Targeted apoptosis induction in cancer", which is in fact the subject of this thesis.

The next five chapters of this thesis are published reports on our novel therapeutic strategy designed to selectively activate apoptosis in cancer cells by targeted delivery of two prominent members of the TNF-superfamily of Death Inducing Ligands, namely TRAIL and FASL. TRAIL and FASL are essential effector molecules that are involved in the targeted elimination of cancerous and virus-infected cells by T- and NK-cells. T-cells selectively eliminate target cells by MHC-restricted activation of apoptosis, which is commenced only after specific recognition, binding, and co-stimulatory immune activation. Unfortunately, the passive transfer of tumour-specific T-cells in cancer therapy has proven to be extremely difficult. However, soluble forms of the effector molecules involved, c.q. soluble-TRAIL (sTRAIL) and soluble FASL (sFASL) have retained residual and promising intrinsic tumour-selective activity in their own right.
However, several potential limitations for the therapeutic application of soluble recombinant forms of TRAIL and FASL have been described. One fundamental problem to be addressed is the widespread expression of the various cognate death receptors throughout the human body. This unfavorable surplus of potential binding sites will hamper tumour-selective accretion of both TRAIL and FASL. In addition, the recombinant soluble forms

of TRAIL and FASL appear less efficacious at inducing apoptosis than the corresponding membrane-bound versions.

The aim of the research described in this thesis was to develop a general strategy that augments the therapeutic value of apoptosis inducing ligands, such as TRAIL and FASL. In **chapter 3** we started this endeavor by designing a recombinant fusion protein, designated scFvC54:sTRAIL, in which sTRAIL was genetically linked to the scFvC54 antibody fragment that specifically targets the Epithelial Glycoprotein-2 (EGP2). EGP2, also known as EpCAM, is a well-established tumour-associated target antigen frequently overexpressed on the cell surface of various human carcinomas. EGP2 has been extensively studied as a target antigen for antibody-mediated imaging and immunotherapy.
Incubation with scFvC54:sTRAIL resulted in strong and selective binding to the cell surface of EGP2-positive cells only. Consequently, the soluble scFvC54:sTRAIL fusion protein was converted into a membrane-bound form, which enabled the efficient crosslinking of agonistic TRAIL receptors 1 and 2. Moreover, the pro-apoptotic signaling observed for scFvC54:sTRAIL was particularly strong as a result of reciprocal apoptosis induction between EGP2-positive target cells. The latter situation closely resembles the way in which the immune response is resolved by fratricide apoptosis induction of activated T-cells.
Of note, it has become evident that selective crosslinking and activation of not only TRAIL-R1 but also of TRAIL-R2 can be of considerable importance for TRAIL-based therapy. Recently, it was demonstrated that apoptosis in certain tumours is predominantly engaged via either TRAIL-R1 or TRAIL-R2. Unlike conventional recombinant sTRAIL preparations, scFvC54:sTRAIL can efficiently induce apoptosis in target cells not only via TRAIL-R1 but also via TRAIL-R2 signaling.
To accelerate clinical application of our fusion proteins we developed a rapid and versatile plasmid-based expression platform that is suitable for eukaryotic expression in industrial-grade production cell lines. Using this platform, stable homogenous scFvC54:sTRAIL trimers were produced in the absence of high-molecular-weight protein aggregates. This absence of aggregates is an important feature of our production system since sTRAIL (and sFASL) aggregates have been implicated in toxicity towards normal human cell types.

A serious limitation of many current antibody-based approaches is the escape of target antigen-negative tumour cells from therapy. Target antigen-negative tumour cells may be pre-existent in the lesion or can arise during or after therapy due to target antigen shedding, masking, or therapy-induced down-regulation. In **chapter 4**, we assessed the so-called bystander effect, predicted as being an essential part of the concept of targeted

apoptosis induction by scFv:sTRAIL fusion proteins (see **chapter 4**, Fig.1, page 62).
The bystander effect is based on the principle that targeted tumour cells are not only eliminated, but are also exploited to convey a therapeutic effect towards neighboring tumour cells that lack expression of the target antigen. For scFvC54:sTRAIL, selective binding to EGP2-positive tumour cells allows for the crosslinking of agonistic TRAIL receptors on neighboring tumour cells, even when they lack EGP2 expression.
To determine the anti-tumour bystander activity of fusion protein scFvC54:sTRAIL, a panel of cell lines was selected representing three major human malignancies, namely acute lymphoblastic T-cell leukemia, B-cell lymphoma, and glioblastoma multiforme, all of which are normally EGP2-negative. By retroviral-transduction, variants of these cell lines that ectopically express EGP2 at the cell surface were generated. Using mixed cultures of parental EGP2-negative bystander cells and EGP2-positive target cells, we showed that, as predicted, selective binding of scFvC54:sTRAIL to EGP2-positive target cells conveyed an exceptionally potent anti–tumour bystander effect in EGP2-negative tumour bystander cells. The anti-tumour bystander activity of scFvC54:sTRAIL was detectable at target-to-bystander cell ratios as low as 1:100 and did not occur in the absence of target cells. Importantly, scFvC54:sTRAIL showed no detectable signs of "innocent" bystander activity towards freshly isolated blood cells. We concluded that the anti-tumour bystander activity of scFv:sTRAIL fusion proteins is a robust feature that may be important in cases where cancer cells escape from targeted therapy due to (partial) loss of target antigen expression.

In **chapters 3** and **4** we exploited EGP2 as a model target antigen, since EGP2 has no intrinsic signaling activity that might interfere or obscure the evaluation of target-cell restricted apoptosis as induced by scFvC54:sTRAIL. In **chapter 5**, we extended our investigations by targeting an antigen of which signaling activity is important in carcinogenesis. As a clinically relevant prototype of such a signaling target antigen we selected the epidermal growth factor receptor (EGFR). Aberrant EGFR signaling has since long been recognized as an important contributor to malignant progression by e.g. enhancing cancer cell growth and increasing resistance of cancer cells to apoptosis. Several strategies designed to inhibit aberrant EGFR-signaling have been developed, including monoclonal antibodies (MAb C225 (Cetuximab) and MAb 425) and small molecule tyrosine kinase inhibitors such as Iressa (also known as ZD1839 or Gefitinib). The clinical efficacy of both classes of EGFR-signaling antagonists relies on multiple anti-cancer mechanisms, including inhibition of cell cycle progression, inhibition of metastatic potential, and an increased susceptibility to apoptosis. Importantly, synergistic tumoricidal effects have been reported upon combination of EGFR-signaling antagonists with recombinant sTRAIL.
Therefore, we constructed a novel scFv:sTRAIL fusion protein, designated scFv425:sTRAIL,

designed to selectively target EGFR and thereby block EGFR mitogenic signaling, while simultaneously triggering TRAIL-receptor-mediated apoptotic signaling.
As expected, *in vitro* treatment with scFv425:sTRAIL resulted in its specific accretion at the cell surface of EGFR-positive cells only and in rapid inactivation of EGFR and downstream mitogenic signaling. As a result, EGFR-positive cells were sensitized to apoptosis. Simultaneously, the TRAIL domain of the scFv425:sTRAIL fusion protein selectively induced apoptosis by crosslinking of agonistic TRAIL-receptors. Similar to scFvC54:sTRAIL, scFv425:sTRAIL conveyed a potent anti-tumour bystander effect towards EGFR-negative bystander tumour cells in mixed culture experiments.
Of note, co-treatment of EGFR-positive tumour cells with the EGFR-tyrosine kinase inhibitor Iressa and scFv425:sTRAIL resulted in a potent synergistic pro-apoptotic effect. Taken together, the favorable characteristics of scFv425:sTRAIL, alone and in combination with Iressa, indicate the potential value of scFv425:sTRAIL for the treatment of EGFR-expressing malignancies.

In **chapter 6**, the feasibility of targeted induction of apoptosis by scFv:sTRAIL fusion proteins in hematological malignancies was assessed. To this end we constructed scFvCD7:sTRAIL, which harbors specificity for the T-cell leukemia-associated antigen CD7. Treatment with scFvCD7:sTRAIL induced CD7-restricted apoptosis in a series of malignant T-cell lines. Importantly, normal resting leukocytes, activated T-cells, and vascular endothelial cells (human umbilical vein endothelial cells) were fully resistant to apoptosis induction by scFvCD7:sTRAIL. Compared to scFvCD7:ETA, a CD7-specific pseudomonas Exotoxin-A immunotoxin, scFvCD7:sTRAIL possessed superior pro-apoptotic activity towards tumour cells. Moreover, scFvCD7:ETA did show toxicity towards normal CD7-positive peripheral blood lymphocytes. *In vitro* treatment of blood cells freshly-derived from T-acute lymphoblastic leukemia patients resulted in marked apoptosis of the malignant T cells that was strongly augmented by the chemotherapeutic agent vincristin.
These pre-clinical data clearly indicate that scFvCD7:sTRAIL is a potent and leukemia-specific therapeutic agent that might be useful in the treatment of CD7-positive acute T-cell leukemia and lymphoma. This novel strategy might help broaden the therapeutic spectrum for T-cell leukemia, since current treatment is predominantly limited to conventional cytotoxic therapy with only limited therapeutic response and significant morbidity.

In **chapter 7**, the concept of targeted delivery of sTRAIL as described and discussed in **chapters 3 - 6** was extended to the targeted delivery of sFASL. Previously, it was reported that recombinant sFASL induced severe liver toxicity in mice, a finding that

precluded its therapeutic application in humans. However, recent evidence indicates that this toxicity is due to contaminating high molecular-weight aggregates present in the respective sFASL preparations. In fact, it was demonstrated that homogeneous trimeric sFASL itself is non-toxic but also lacks tumoricidal activity. Consequently, new sFASL-based anti-cancer strategies have to meet the criterion of strictly localized activation of sFASL at the tumour cell surface. To test the feasibility of meeting these criteria, the sTRAIL domain from scFvCD7:sTRAIL (see **chapter 6**) was exchanged for sFASL, yielding fusion protein scFvCD7:sFASL. Fusion protein scFvCD7:sFASL proved to be biologically inactive as a soluble homotrimer and acquired tumoricidal activity only after specific binding to tumour cell surface-expressed CD7, as evidenced by its potent CD7-restricted activity on T-ALL cell lines and patient-derived T-ALL, PTCL, and CD7-positive AML cells. In addition, apoptosis induction by scFvCD7:sFASL was augmented by various conventional and experimental drugs, including proteasome inhibitor Velcade. Importantly, normal human lymphocytes and endothelial cells were resistant to treatment, while in activated T-cells only moderate induction of apoptosis was detected. Together, these results establish the feasibility of localized activation of sFASL at the tumour cell surface, which may rekindle the interest in application of sFASL in tumour therapy.

In **chapter 8**, a comprehensive summary of the results and conclusions is provided along with perspectives for further development of the deliberate and selective induction of apoptosis in the treatment of human cancer.

Targeted induction of apoptosis for cancer therapy

current progress and prospects

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chapter 2

Abstract

Important breakthroughs in cancer therapy include clinical application of antibodies, such as Rituximab, and small inhibitory molecules, such as Iressa and Velcade. In addition, recent reports have indicated the therapeutic potential of physiological pro-apoptotic proteins such as TRAIL and Galectin-1. Although unrelated at first glance, each strategy relies on the deliberate and selective induction of apoptosis in malignant cells. Importantly, therapy-resistance in cancer is frequently associated with de-regulation in the mechanisms that control apoptosis. However, cancer cells are often reliant on these molecular aberrations for survival. Therefore, selective induction of apoptosis in cancer cells but not normal cells seems feasible. Here, we review recent progress and prospects of selected novel anti-cancer approaches that specifically target and sensitize cancer cells to apoptosis.

Selective activation of apoptosis in cancer cells

Apoptosis is an elaborate cellular homeostasis mechanism that ensures correct development and function of multicellular organisms. In this respect, the immune system is perfectly equipped to target apoptosis selectively towards cells with potentially dangerous phenotypes. The immune system uses an enormous repertoire of highly selective receptors (e.g. on T and B cells) combined with various potent pro-apoptotic effector mechanisms [e.g. granzymes and fibroblast-associated cell surface (FAS) ligand (FASL)]. However, during tumor progression cancer cells can develop ingenious mechanisms to escape from the immune system – most notably, an increased resistance to apoptosis.

Recent detailed knowledge on molecular aberrations that underlie carcinogenesis has identified various possible targets for therapeutic intervention in cancer. As a result, a plethora of novel targeted approaches has been designed. A common denominator for many of these approaches is the elimination of cancer cells by the preferential induction of apoptosis in these cells while sparing normal cells.

Here, we briefly introduce the normal molecular pathways that underlie apoptosis (Fig.1) and some of the major defects observed in cancer cells. We also highlight recent advances in therapeutic approaches that have been designed to exploit differences between cancer and normal cells to tip the balance of cellular fate towards apoptotic cell death in a selective way (Fig.2). We review some of the most promising approaches that selectively target cancer associated cell-surface molecules. We then deal with strategies that aim to induce apoptosis on the basis of intracellular pathways that might be altered in cancer cells. Subsequently, we review novel approaches that selectively exploit cancer-related alterations in the nucleus to induce apoptosis. In table 1, the respective target, the

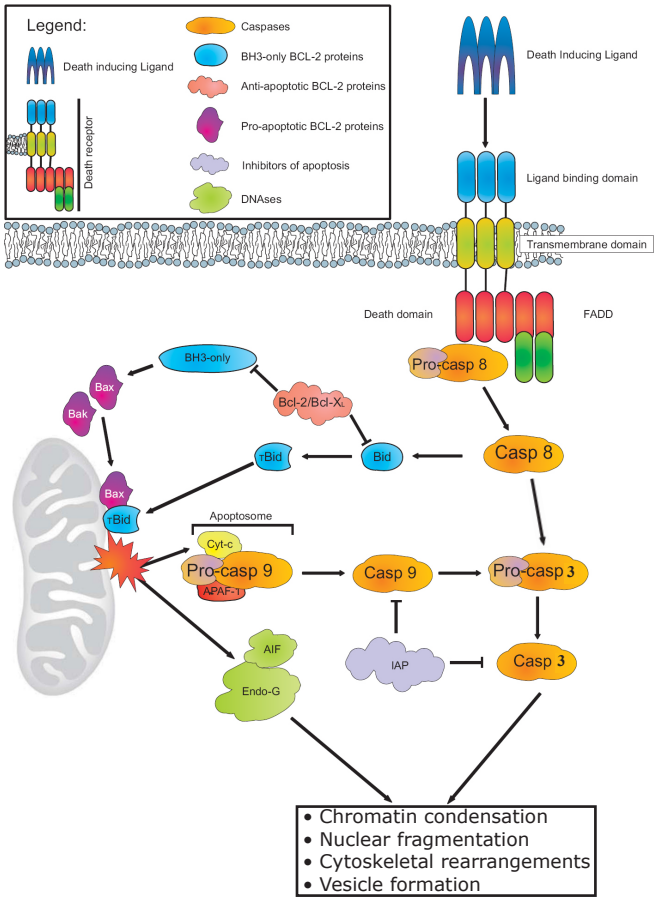

Fig.1. The molecular mechanisms of apoptosis. The mitochondrial pathway of apoptosis is controlled by the BCL-2 family of pro- and anti-apoptotic proteins. When activated, the upstream sensors of intracellular stress – the BH3-only proteins (e.g. BID) – associate with the pro-apoptotic BCL-2 proteins BAX or BAK and translocate to the outer mitochondrial membrane. Subsequently, pores are formed in this membrane, resulting in the release of, among others, the DNases apoptosis inducing factor (AIF) and endonuclease G, both of which translocate to the nucleus and induce DNA fragmentation. In addition, cytochrome c is released from the mitochondria and associates with APAF-1 and pro-caspase-9 into the so-called apoptosome. In the apoptosome, caspase-9 is proteolytically processed into its active form, whereupon effector caspases (e.g. caspase-3) can be activated. BH3-only proteins are inhibited by the anti-apoptotic BCL-2 proteins, such as BCL-2 and BCL-xL, that bind to and inhibit the association of BH3-only proteins with BAX or BAK. The death-receptor pathway of apoptosis is activated upon interaction of a death receptor with its cognate death-inducing ligand, resulting in recruitment of the adaptor protein FADD and pro-caspase-8 to the intracellular death domain of the receptor. Concomitantly, pro-caspase-8 is proteolytically cleaved and activates the effector caspases. In addition, caspase-8 can cleave the BH3-only protein BID, thereby activating a mitochondrial amplification loop. Abbreviations: casp, caspase; cyt c, cytochrome c; pro-casp, pro-caspase.

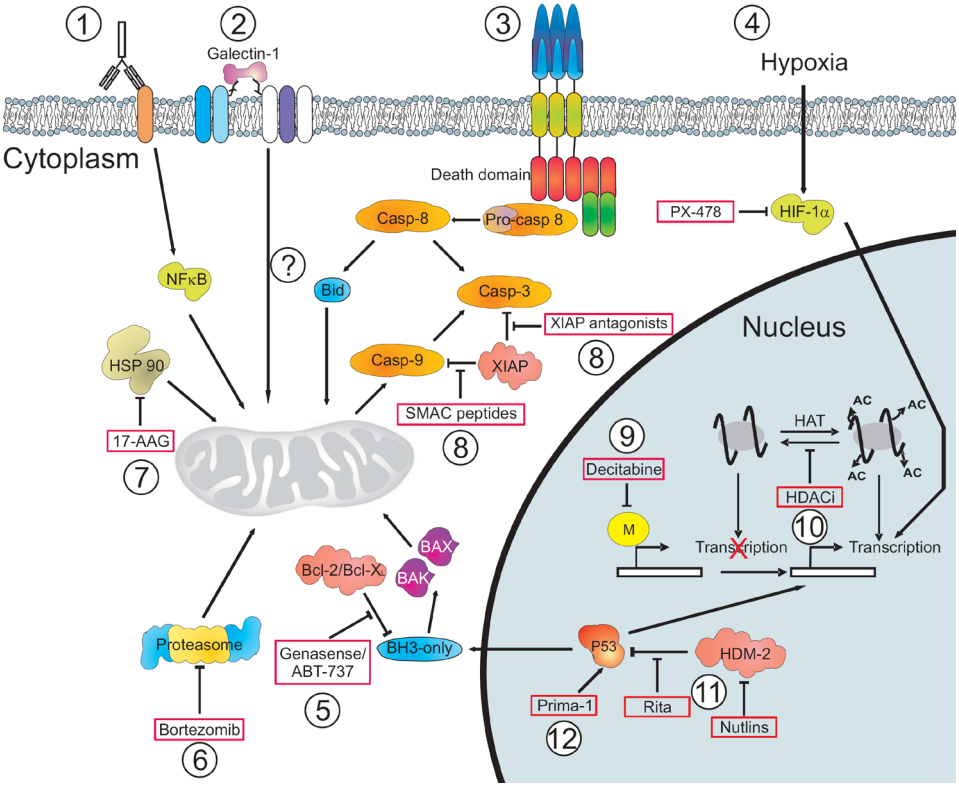

Fig.2. Therapeutic strategies for the targeted induction of apoptosis in human cancer cells. (1) Naked monoclonal antibodies (e.g. RituxiMAb and ApolizuMAb) bind to and crosslink their target antigen, which results in activation of the mitochondrial pathway of apoptosis. (2) Galectin-1 binds to carbohydrate moieties on various cell surface-expressed proteins and triggers caspase-independent apoptosis that is characterized by specific release of endonuclease G from the mitochondria. (3) Apoptosis is activated by triggering of death receptors by recombinant forms of the cognate death-inducing ligand. (4) Hypoxia in the tumor microenvironment upregulates the expression of the transcription factor HIF-1α, which protects towards apoptosis. The function of HIF-1α can be specifically inhibited by the small-molecule inhibitor PX-478, whereupon cells undergo apoptosis. (5) The anti-apoptotic proteins BCL-2 and BCL-xL shift the balance of mitochondria towards survival. Inhibition of BCL-2, using the antisense ODN genasense, and inhibition of both BCL-2 and BCL-xL, using small molecule inhibitor ABT-737, blocks the anti-apoptotic function of these proteins and thereby shifts the balance towards induction of mitochondrial apoptosis. (6) Inhibition of the proteasome, using the inhibitor bortezomib, deregulates protein homeostasis leading to cell cycle arrest and activation of the mitochondrial apoptotic pathway. (7) Inhibition of the important chaperone molecule HSP90, using 17-AAG, results in proteasomal degradation of regulatory proteins and subsequent cell-cycle arrest and activation of the mitochondrial apoptotic pathway. (8) Inhibition of the XIAP-mediated block on active caspase-9, using SMAC peptides, sensitizes tumor cells towards apoptosis. Inhibition of the XIAP-mediated block on active caspase-3, using small-molecule inhibitors, results in potent activation of apoptosis. (9) Demethylation of cellular DNA, using decitabine, induces growth arrest and concomitant apoptosis. (10) Restoring the balance between histone acetylation and histone deacetylation with histone deacetylase inhibitors such as valproic acid results in increased acetylation,

whereby transcription of a finite number of genes is up- or downregulated. Subsequently, cancer cells undergo cell-cycle arrest followed by apoptosis. (11) Inhibition of the interaction of wild-type p53 with its negative regulator HDM-2 by blocking the HDM-2–p53 interaction site on HDM-2, using Nutlins, or by blocking the HDM-2–p53 interaction site on p53, using RITA, upregulates p53 target genes and potently induces apoptosis. (12) Re-activation of mutant p53, using PRIMA-1, upregulates p53 target genes and potently induces apoptosis. Abbreviations: casp, caspase; pro-casp, pro-caspase.

stage of development and clinical status of these therapeutic strategies are summarized. Finally, we discuss directions to integrate these approaches in an attempt to design selective tumoricidal pro-apoptotic strategies with low or non-overlapping toxicity towards normal cells.

Molecular pathways of apoptosis and cancer-specific defects

Central to the execution of apoptosis is the coordinated activation of a subset of caspases – executioner caspases – that cleave multiple cellular substrates, ultimately resulting in apoptotic cell death (Fig.1). These executioner caspases (caspase-3, caspase-6 and caspase-7) are themselves activated by so-called initiator caspases. All caspases are produced as inactive pro-enzymes and are activated by proteolytic processing.

In most physiological situations, apoptosis is initiated via the mitochondrial pathway. Central to this pathway is the permeabilization of the outer mitochondrial membrane with subsequent release of several pro-apoptotic factors into the cytosol. One of these factors, cytochrome-c, alters the conformation of the cytosolic protein apoptotic protease activating factor-1 (APAF-1), whereupon this protein oligomerizes with pro-caspase-9 into the so-called apoptosome. Pro-caspase-9 is then autoproteolytically processed and, subsequently, activates effector caspases.

Mitochondrial sensitivity to apoptosis is exquisitely regulated by the B-cell leukemia/lymphoma 2 (BCL-2) family of pro- and anti-apoptotic proteins[1]. These proteins are defined in part by homology shared within four conserved BCL-2 homology (BH) domains. Anti-apoptotic members, such as BCL-2 and BCL-xL, are conserved in all four BH domains, whereas the multidomain pro-apoptotic members BCL-2-associated X protein (BAX) and BCL-2 antagonistic killer (BAK) show conservation in BH1–BH3 domains. The BH3-only proteins of this family are thought to serve as upstream sensors that respond to diverse death signals. BH3-only proteins seem to require BAX or BAK for their pro-apoptotic activity and are sequestered by anti-apoptotic BCL-2 proteins. Interaction of BH3-only proteins with BAX and/or BAK is prevented by anti-apoptotic BCL-2 proteins, which, consequently, restrains initiation of mitochondrial apoptosis.

The second main pathway for apoptosis – the death receptor pathway – has a fundamental role in maintenance of tissue homeostasis, especially in the immune system. This pathway is activated upon interaction of death receptors at the cell surface with their cognate ligands on, for example, T cells, whereupon the adaptor protein FAS-associated death

Table 1: Cellular target, effector mechanism and (pre)clinical status of selected apoptosis-inducing therapeutic compounds

Therapeutic compound	Cellular target	Mechanism of apoptosis induction	Clinical status	Clinical effects	Refs
Immunotoxins					
Gemtuzumab ozogamicin	CD33	calicheamicin-mediated DNA-damage	FDA approved (AML)		6
Inotuzumab ozogamicin	CD22	calicheamicin-mediated DNA-damage	in phase I trial (NHL)	?	7
CD22-Exotoxin-A	CD22	Pseudomonas Exotoxin-A mediated DNA-damage	completed phase I trial (NHL)	CR; 66%, PR; 19%	8
Antibodies					
Rituximab	CD20	cross-linking of CD20; mitochondrial apoptosis	FDA approved (NHL)		9
Apolizumab	HLA-DR	cross-linking of HLA-DR; ROS generation; mitochondrial apoptosis	in phase I trial (NHL)	?	11
HGS-ETR-1	TRAIL-R1	activation of DR-apoptosis by cross-linking of TRAIL-R1	in phase I trial	?	28
HGS-ETR-2	TRAIL-R2	activation of DR-apoptosis by cross-linking of TRAIL-R2	in phase I trial	?	29
Death Inducing ligands					
TNF	TNF receptors	DR-apoptosis by TNF-R activation	FDA approved (isolated limb/liver perfusion)	CR	14
FasL	Fas	DR-apoptosis by Fas activation	pre-clinical (proven efficacious in animal models)	n.a	15
TRAIL	TRAIL receptors	DR-apoptosis by TRAIL-R activation	pre-clinical (proven efficacious in animal models)	n.a	17
scFv:sFasL	Fas	DR-apoptosis by Fas activation	pre-clinical (proven efficacious in animal model)	n.a	24
scFv:sTNF-TNF-R	TNF receptors	DR-apoptosis by TNF-R activation	pre-clinical (in vitro)	n.a	79
lectins					
Galectin-1	not characterized	Growth arrest followed by caspase-independent apoptosis	pre-clinical (proven efficacious in animal models)	n.a	34
Galectin-3	not characterized	Growth arrest followed by caspase-dependent apoptosis	pre-clinical (in vitro)	n.a.	38
Intra-cellular targets					
Bortezomib	Proteasome	Inhibition of proteasome, activating mitochondrial apoptosis	FDA approved (multiple myeloma)	PR; 5%, SD; 50%	40
Genasense	BCL-2	Inhibition of Bcl-2, shifting balance to mitochondrial apoptosis	completed phase I trial (advanced coloncarcinoma)	n.a.	44
ABT-737	BCL-2/BCL-xL	Inhibition of Bcl-XL, shifting balance to mitochondrial apoptosis	pre-clinical (proven efficacious in animal model)	n.a.	46
xxx	XIAP	Inhibition of XIAP-block on active caspase-3	pre-clinical (proven efficacious in animal model)	SD; 7%	4
17-AAG	HSP-90	Growth arrest followed by caspase-dependent apoptosis	completed phase I trial (advanced cancer)	n.a.	51
Nutlins/RITA	p53/HDM-2 interaction	reactivates p53 transcription; activates mitochondrial apoptosis	pre-clinical (proven efficacious in animal model)	n.a.	57;58
PRIMA-1	mutant p53	reactivates mutant p53; activates mitochondrial apoptosis	pre-clinical (proven efficacious in animal model)	n.a.	59
PX-478	HIF-1α	inhibits HIF-1α transcription, sensitizes cells to apoptosis	pre-clinical (proven efficacious in animal model)	n.a.	62
Antisense HIF-1α	HIF-1α	inhibits HIF-1α transcription, sensitizes cells to apoptosis	pre-clinical (proven efficacious in animal model)	n.a.	63
Epigenetic regulation					
Decitabine	DNA-methylation	DNA-methylation; activation of mitochondrial apoptosis	completed phase II trial (CLL)	CHR; 34%, PHR; 20%	66
HDACi	Histone deacetylases	HDAC-inhibition, activation of mitochondrial apoptosis	comleted phase I/II clinical trials		69

[a] Abbreviations: 17-AAG, 17-allylamino-17-demethoxygeldanamicyn; AML, acute myeloid leukemia; BCL, B-cell leukemia/lymphoma; CHR, complete hematological response; CLL, chronic lymphocytic leukemia; CR, complete response; DR, death receptor; FAS, fibroblast-associated cell surface; FASL, FAS ligand; FDA, Food and Drug administration; GO, gemtuzuMAb ozogamicin; HDAC, histone deacetylase; HDACi, HDAC inhibitior; HDM-2, human double minute-2; HIF-1α, hypoxia-inducible factor-1α; HLA-DR, humanleukocyte-antigen-DR; HSP90, heat shock protein 90; MDS, myelodysplastic syndrome; OR, objective responders; N.a., not available; NHL, non-Hodgkin's lymphoma; PHR, partial hematological response; PR, partial response; PRIMA-1, p53-dependent reactivation and induction of massive apoptosis-1; RITA, reactivation of p53 and induction of tumor-cell apoptosis; ROS, reactive oxygen species; SD, stable disease; sFASL, soluble FAS ligand; sTNF, soluble tumor necrosis factor; TNF; tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; XIAP, X-linked inhibitor of apoptosis protein.

domain (FADD) and initiator caspase-8 or caspase-10 are recruited to the intracellular death domains of these receptors. Assembly of this so-called death-inducing signaling complex (DISC) leads to sequential activation of initiator and effector caspases and, ultimately, results in apoptotic cell death. In some cells, the death-receptor pathway relies on a mitochondrial amplification loop that is activated by caspase-8-mediated cleavage of the BH3-only interacting domain death agonist BID to a truncated form. Truncated BID subsequently activates the mitochondrial pathway. An important regulator of the death-receptor pathway is the caspase-8 homologue cellular FAS-associated death-domain-like interleukin-1b-converting enzyme (FLICE) inhibitory protein (c-FLIP), which competes with and inhibits autocleavage of caspase-8.

Once activated, caspases are subject to regulation by the family of inhibitor of apoptosis proteins (IAPs)[2]. The IAP family represents an integral checkpoint in the execution of apoptosis by their ability to bind to and inhibit activated caspases, thereby halting the execution phase of apoptosis.

Many cancers are characterized by inactivating mutations in pro-apoptotic proteins; for example, in the tumor suppressor p53, which is instrumental in the activation of the mitochondrial pathway of apoptosis upon DNA damage by substances such as chemotherapeutics[3]. Alternatively, anti-apoptotic proteins such as BCL-2 and IAP family members are frequently upregulated. These cancer-cell specific aberrations enable evasion of apoptosis and also confer resistance to chemotherapeutics that typically work by induction of apoptosis. However, cancer cells are reliant on these aberrations for survival, as evidenced by recent findings that have indicated that these cells paradoxically are more prone to apoptosis than normal cells[4] and can even have 'ready to go' active effector caspases[5]. However, owing to genetic instability, a population of cancer cells arises in which the apoptosis-prone phenotype is over-ruled by upregulation of various anti-apoptotic mechanisms. It has been proposed that malignant transformation induces intracellular stress signals in an attempt of the cancerous cell to self-terminate. Therefore, selective induction of apoptosis in cancer cells but not in normal cells seems feasible. In recent years, identified molecular aberrations have been the target of novel strategies; some of the most promising will be discussed in this review.

Targeted induction of apoptosis using antibodies

Compared to their normal counterparts, cancer cells often display a qualitatively and/or quantitatively different repertoire of cell-surface molecules that can be selectively targeted in cancer therapy. Most established strategies for targeted therapy are based on cancer-cell-selective monoclonal antibodies (MAbs). Often, the tumoricidal effect of antibody-based therapy relies on highly toxic and proapoptotic compounds directly conjugated to antibodies that potently activate apoptosis upon internalization and processing. Although

such toxin-based strategies have often been hampered by toxicity and immunogenicity, a better target validation and the development of strategies to reduce immunogenicity have provided some promising results. For example, the immunoconjugate gemtuzumab ozogamicin (GO) has recently been the first immunotoxin to be clinically validated. GO comprises a humanized anti-CD33 antibody linked to a derivative of calicheamicin[6]. Calicheamicin is an antibiotic isolated from Micromonospora echinospora that is 1000-fold more potent than conventional chemotherapeutics. Calicheamicin induces sequence-specific double-strand DNA breaks, thereby activating apoptosis. GO induces objective responses when used as a single agent in acute myeloid leukemia, but is associated with serious hepatic veno-occlusive disease. A similar CD22-targeted immunoconjugate, inotuzumab ozogamicin[7], is currently being evaluated in Phase I clinical trials for non-Hodgkin's lymphoma. Furthermore, in a recent phase I clinical trial with a CD22-targeted immunotoxin that contains *Pseudomonas* exotoxin A, a complete response rate of 61% was reported[8].

Naked chimeric or fully human antibodies have also shown remarkable anti-cancer activity predominantly by recruiting the patient immune effector mechanisms such as complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). However, at least part of the tumoricidal activity of certain antibodies originates from direct activation of apoptosis by crosslinking of the respective target antigen. For example, the chimeric anti-CD20 antibody Rituximab inhibits nuclear factor-κB (NF-κB) signaling and potently activates the mitochondrial pathway of apoptosis by crosslinking of cell-surface-expressed CD20[9]. This finding might also be relevant for the efficacy of Rituximab in vivo[10]. A similar observation has been made for Apolizumab, a humanized anti-human-leukocyte-antigen-DR (HLA-DR) b-chain-specific antibody, which shows potent pro-apoptotic activity in chronic lymphocytic leukemia that depends on cytoskeletal rearrangements and formation of reactive oxygen species (ROS)[11].

Because better strategies for target validation and reduction of immunogenicity have been developed, the promise held by antibody-based approaches for >20 years is slowly turning into reality. However, several issues still remain to be addressed, one of which is the known antigen heterogeneity of tumor cells. Tumor cells that downregulate or lose target-antigen expression can easily escape from antibody-based targeted therapy (e.g. Rituximab). Combinatorial strategies that target multiple antigens is one of the ways to overcome this problem. In addition, approaches have been developed to take advantage of the so-called 'bystander effect', which is based on the principle that targeted tumor cells are not only eliminated but also exploited to convey the therapeutic effect towards neighboring tumor cells devoid of target-antigen expression. Bystander effects have been reported for several antibody-based therapeutic approaches[12,13], but in most cases require consecutive processing steps, including drug internalization, enzymatic conversion and

inter-cellular communication (e.g. via gap junctions) between target and bystander cells. These consecutive processing steps can still pose severe limits to the bystander effect because tumor cells in which one or more of these steps are inhibited or absent will be resistant to the bystander effect.

Apoptosis by activation of members of the tumor necrosis factor (TNF) receptor family

The direct activation of the apoptotic machinery in cancer cells using recombinant soluble forms of tumor necrosis factor (TNF), FASL and TNF-related apoptosis-inducing ligand (TRAIL) has attracted much attention. TNF, FASL and TRAIL, three major immune effector molecules, all possess high tumoricidal pro-apoptotic activity.

However, severe cardiovascular toxicity has limited the therapeutic use of soluble TNF (sTNF) to loco-regional applications, such as isolated limb perfusion, where it has shown impressive clinical responses in combination with conventional chemotherapy[14].

The therapeutic use of soluble FASL (sFASL) was originally deemed impossible owing to severe toxicity in mice[15]. However, this toxicity has recently been attributed to contaminating multimeric or aggregated forms of sFASL. Indeed, as little as two adjacent trimeric FASLs are sufficient for activating FAS-apoptotic signaling. MegaFASL, a hexameric FASL preparation, showed potent cytotoxic effects on some human malignant hematopoietic cells[16]. Its toxicity towards normal lymphocytes remains to be determined. By contrast, homogeneous trimeric sFASL preparations are devoid of toxicity but are also devoid of tumoricidal activity. We and others have developed promising strategies to restore the activity of sFASL trimers only after tumor-selective delivery as will be discussed later.

TRAIL has generated tremendous enthusiasm because it selectively induces apoptosis in various malignant cell types, but not in normal cells[17]. However, the clinical efficacy of soluble TRAIL (sTRAIL) might be hampered because of the widespread expression of different TRAIL receptors throughout the body. In addition, several tumor types express higher levels of TRAIL-receptor-2 (TRAIL-R2) than of TRAIL-receptor-1 (TRAIL-R1), whereas TRAIL-R2 signaling is only poorly activated by sTRAIL[18]. Intriguingly, in some solid tumor types that express equal levels of TRAIL-R1 and TRAIL-R2, apoptosis was reported to be selectively mediated by TRAIL-receptor-2 signaling[19]. Conversely, apoptotic signaling in chronic myeloid leukemia is mediated exclusively by TRAIL-R1 signaling[20]. This notion has led to the development of TRAIL mutants with selectivity for either TRAIL-R2 or TRAIL-R1[19,20].

Recently, we and others have demonstrated that the tumor-selective binding of sTRAIL and sFASL can be strongly enhanced by genetic fusion to a tumor-selective antibody fragment[21–25]. The binding of such fusion proteins to cell-surface-expressed target antigens

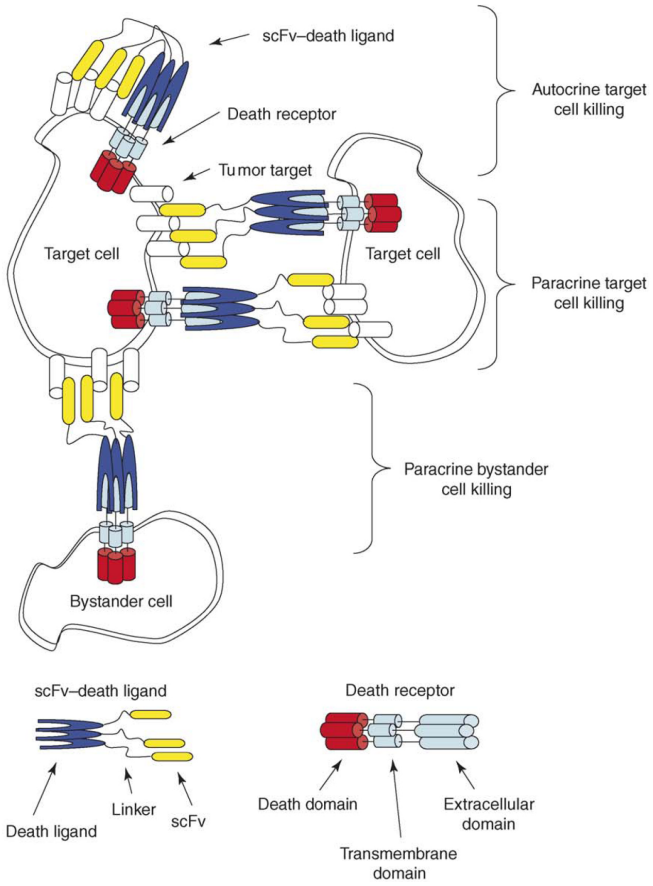

Fig.3. Target-cell-restricted induction of apoptosis by scFv–death-ligand fusion proteins. Specific binding of scFv–death-ligand fusion proteins to the tumor target antigen results in accretion at the cell surface. Subsequently, apoptosis can be induced in an autocrine manner by binding to the cognate death receptor on the same tumor cells. Alternatively, specific binding of scFv–death ligand to the tumor target antigen on one cell can induce crosslinking of cognate death receptors on a neighboring target antigen-positive tumor cell, resulting in paracrine target cell apoptosis. In addition, paracrine crosslinking of cognate death receptors on a neighboring target antigen negative tumor cell results in bystander cell apoptosis. This bystander effect depends only on the presence of functional cognate death receptors.

converts the soluble death ligands into membrane-bound molecules capable of crosslinking agonistic death receptors in an autocrine and paracrine manner (Fig.3). In this way also neighboring tumor cells devoid of target antigen can be effectively eliminated by the so-called bystander effect[26,27]. In this case, the bystander effect solely depends on accretion of fusion proteins to the cell surface of targeted cells and does not require additional

cellular processing other than intact death-receptor signaling pathways. Proof of principle for this approach has been obtained for sTRAIL and sFASL in both solid tumors[21–24] and leukemia[25,27], with no or minimal activity towards normal cells.

Activation of TRAIL receptors has also been pursued using agonistic MAbs, of which HGS-ETR-1 and HGS-ETR-2 are currently evaluated in clinical trials[28,29]. An important difference between these TRAIL-receptor selective MAbs and TRAIL is the fact that TRAIL interacts with both its agonistic receptors and its decoy receptors. This might give TRAIL either a wider or a narrower and more unpredictable therapeutic window than that of TRAIL-receptor-selective MAbs. Intriguingly, a recent report has indicated that a mouse agonistic TRAIL-R2 MAb also induced potent tumor-specific T-cell immunity[30]. As a result, a second challenge with the same tumor cells that were engineered to overexpress c-FLIP could still be eliminated. This strategy might thus be of potential value to overcome acquired TRAIL resistance[31]. However, epigenetic silencing of TRAIL-receptor expression might pose another daunting challenge to be overcome[32].

In conclusion, because much of the molecular pathways of TNF, FASL and TRAIL has been elucidated, the therapeutic potential of these ligands in cancer has been firmly established in pre-clinical studies. However, cancer-cell selective activation remains an issue to be thoroughly addressed to use these molecules for clinical applications.

Activation of apoptosis by modulating Galectins

Recently, the physiologically occurring anti-proliferative galectins were shown to have promising anti-tumor activity[33]. Galectins are a family of lectins with affinity for β-galactoside residues of cell-surface glycoproteins expressed by both normal and cancer cells. However, upon binding, regulatory functions to which normal and cancer cells respond differently are enforced. For example, galectin-1 blocks the cell cycle in late S-phase by altering the expression of cell-cycle controller proteins such as the transcription factor E2F1[34]. Intriguingly, in tumor cells this S-phase block is followed by activation of apoptosis due to constitutively high E2F1 levels, whereas in normal cells there is only growth arrest. Several tumor types, including multi-drug resistant tumor cells, are highly sensitive to apoptosis induction by galectin-1[34–36]. The galectin-1 mediated apoptotic pathway is still largely undefined but is reported to be caspase-independent and to involve the specific release of endonuclease G from the mitochondria[35].

Intriguingly, galectins can have contradictory roles in tumor development[33]. Tumors that ubiquitously express galectin-1 can modulate the anti-tumor immune response by eliminating tumor-infiltrating T cells[37]. In addition, galectins are involved in tumor metastasis by regulation of tumor-cell adhesion and invasiveness. Selective inhibition of cancer cell-expressed Galectin-1 and Galectin-3 has recently been explored using synthetic lactulose amines. This inhibition results in tumor cell-selective apoptosis induction[38].

Therefore, patient-tailored therapy using either recombinant forms of galectin-1 or, alternatively, inhibitors of endogenous galectin-1 or galectin-3, depending on the tumor, might be a promising approach to induce apoptosis in a cancer-selective way.

Intracellular activation of apoptosis

Apoptosis by proteasome inhibition

Protein homeostasis is pivotal to cell survival and is mainly regulated by the ubiquitin–proteasome pathway (UPP)[39], which controls the half-life of the majority of cellular proteins. Inhibition of the UPP in cancer cells has yielded promising results. This has been highlighted by the recent approval of the proteasome inhibitor bortezomib (Velcade) for the treatment of multiple myeloma[40]. An important feature of bortezomib is the differential response of normal and cancer cells[41], the basis of which is still a mystery. It has been shown that after bortezomib treatment both normal and cancer cells are growth arrested in the G2–M phase of the cell cycle[41]. However, cancer cells are eliminated by an as yet incompletely characterized apoptotic pathway that converges on mitochondria, whereas normal cells resume division after treatment. Apoptosis by bortezomib is characterized by stabilization of p53[42] and upregulation of the BH3-only protein NOXA[41]. Bortezomib potently augments the apoptotic activity of other therapeutics (e.g. TRAIL[43]) that does not depend on p53 status or BAX expression.

Apoptosis by inhibition of anti-apoptotic BCL-2 family members

Central to the control of the mitochondrial pathway of apoptosis is the balance between pro- and anti-apoptotic members of the BCL-2 family[1]. In cancer, anti-apoptotic proteins such as BCL-2 and BCL-xL are frequently overexpressed, thus shifting the balance towards cell survival. Therefore, therapeutic inhibition of these endogenous inhibitors of apoptosis is an attractive approach that has recently been explored using the antisense oligonucleotide genasense directed against BCL-2[44].

Additionally, the interaction of BCL-2 with BAX and/or BAK has been targeted using small-molecule mimetics that fit the groove on BCL-2 where these pro-apoptotic members bind[45]. More recently, a small-molecule mimetic for BCL-2 and BCL-xL, ABT-737, has been reported to have high-affinity inhibition for this anti-apoptotic protein[46]. ABT-737 induced complete tumor regression in 77% of treated mice with no secondary tumor outgrowth in xenograft models. However, some cell lines were resistant to ABT-737, possibly because of overexpression of other anti-apoptotic BCL-2 family members, such as myeloid-cell leukaemia factor (MCL-1)[47]. Taken together, therapeutic inhibition of these endogenous inhibitors of apoptosis holds great promise for tipping the balance towards apoptosis in cancer. Because various anti-apoptotic BCL-2 members are known to be upregulated, the future of this approach is likely to involve combination strategies that inhibit multiple anti-

apoptotic BCL-2 family members, such as the use of bi-specific BCL-2–BCL-xL antisense oligonucleotide[48].

Apoptosis by inhibition of the X-linked inhibitor of apoptosis protein (XIAP)

Inhibition of activated caspases by IAPs is an integral checkpoint during apoptosis[2]. The first identified member, the X-linked inhibitor of apoptosis protein (XIAP), is upregulated in various cancers and can inhibit both initiator caspase-9 and effector caspase-3. The so-called Baculovirus IAP repeat (BIR)3 domain of XIAP inhibits caspase-9, whereas the BIR2 domain together with the N-terminal linker inhibits caspase-3. Relieving the XIAP-mediated block on caspase-9 was pursued using peptides that contain a BIR3-domain binding motif derived from the XIAP-neutralizing protein second mitochondrial activator of caspases (SMAC). These peptides did not induce apoptosis as single agents, but synergized the apoptotic activity of chemotherapeutic agents and TRAIL. More recently, one of these peptides that specifically relieved the block on not only caspase-9 but also caspase-3 was shown to activate apoptosis as a single agent depending on the expression level of IAPs[49].

Intriguingly, inhibition of the XIAP–caspase-3 interaction using selected polyphenylurea compounds potently and selectively induced apoptosis in cancer cells but not in normal cells[4]. These results strongly indicated that cancer cells are intrinsically more prone to apoptosis than normal cells probably owing to the presence of 'ready to go' processed effector caspases. Indeed, processed active caspase-3 has been detected in tumor cell lines and tumor tissue[5]. Therefore, the inhibition of XIAP and other IAP family members such as survivin can help selectively restore sensitivity to apoptosis in cancer cells, which might be especially useful in combination with other cancer-selective pro-apoptotic approaches, such as TRAIL and p53 reactivation as described below.

Apoptosis by inhibitors of heat shock protein 90 (HSP90)

The activity of many proteins that are involved in carcinogenesis depends on heat shock protein 90 (HSP90) for their maturation and stability[50]. HSP90 is part of a large multichaperone complex that exists in two configurations. In the first configuration, known as open or intermediate, client proteins are loaded. This complex switches to a closed or mature state upon ATP-binding and hydrolysis, resulting in substitution of original co-chaperones with other co-chaperones that help maintain the protein in an active configuration and direct protein maturation. Several HSP-90 inhibitors can lock this multichaperone complex in the open state, leading to proteasomal degradation of client proteins. Most prominent of these is 17-allylamino-17-demethoxygeldanamicyn (17-AAG), which has shown promising activity in Phase I clinical trials[51]. 17-AAG induces growth arrest and apoptosis (e.g. by degradation of B-RAF[52] or 70 kDa zeta-associated

protein ZAP-70[53]). Importantly, it has recently been shown that inhibition of HSP90 abrogates the TNF-induced NF-κB activation by preventing formation of the normally occurring IκB-kinase (IKK) complex[54]. Therefore, combination of HSP90 inhibition and TNF inhibition might be a promising therapeutic approach, as evidenced by the recently described synergistic activation of apoptosis by 17-AAG and TNF[55].

Activation of apoptosis in the nucleus

Apoptosis by restoring p53 activity

The tumor-suppressor p53 is instrumental in the cellular response to stress signals and is crucial in the prevention of tumor development and the success of various anti-cancer strategies[56]. Over 50% of tumors possess inactivating mutations in p53, whereas in tumors that retain wild-type p53 its function is often impaired as a result of overexpression of the negative regulator human double minute-2 (HDM-2). HDM-2 binds to p53 and, consequently, p53 is subject to rapid proteasomal degradation. Therefore, the restoration of p53 activity is a potentially promising therapeutic approach. Several HDM-2-selective inhibitors have been designed to treat tumor cells that express wildtype p53. A recently developed class of HDM-2 inhibitors, the so-called Nutlins[57], disrupt the interaction between wild-type p53 and HDM-2 by binding to the HDM-2–p53 binding pocket on HDM-2. Nutlins potently and tumor-specifically activate apoptosis without toxicity. Conversely, the reactivation of p53 and induction of tumor-cell apoptosis (RITA) inhibits the HDM-2–p53 interaction by binding to p53[58] and, similarly, induces massive apoptosis in p53 wild-type tumor types.

In tumors that express p53 mutant, reactivation of p53 has been attempted using activating compounds such as p53-dependent reactivation and induction of massive apoptosis-1 (PRIMA-1)[59]. These compounds restored transcriptional activity of mutant p53, resulting in potent induction of apoptosis.

In conclusion, the central role of p53 in therapeutic apoptosis induction highlights the rationale to restore its full functionality in cancer cells to overcome therapy resistance. Rational combinatorial strategies of p53 reactivation with, for example, XIAP inhibition might also help to lower the threshold for apoptosis induction specifically in cancer cells.

Apoptosis by inhibition of hypoxia inducible factor-1α (HIF-1α)

Hypoxia in the tumor microenvironment contributes to malignant progression and protects cancer cells from drug-induced apoptosis[60]. Hypoxia-inducible factor-1α (HIF-1α) is a pivotal component in the hypoxia response during tumor development. The effect of HIF-1α upregulation on solid-tumor growth is a balance between reduced cell proliferation and enhanced survival, the latter being proportionally greater[61]. Recently, a small molecule inhibitor of HIF-1α, named PX-478, has been described to have promising tumoricidal

effects in xenograft tumor models[62]. The anti-tumor effect of PX-478 positively correlated with tumoral HIF-1α levels and was accompanied by massive apoptosis. Additionally, anti-sense DNA strategies directed against HIF-1α have been designed, which revealed potent synergistic activity in combination with chemotherapy[63]. These data clearly indicate that modulation of molecules or pathways involved in tumor hypoxia can restore sensitivity to therapeutic apoptosis induction.

Epigenetic regulation of apoptosis

Apoptosis by DNA-methylase inhibitors

DNA methylation has a regulatory role in gene expression during normal development but can also mediate epigenetic silencing of genes in cancer[64]. Many individual genes including tumor suppressors have been shown to undergo de novo methylation in specific tumor types. Specific DNA methyltransferases methylate DNA at the carbon-5 position of cytosine. An important example is the methylation of the E-cadherin promoter, which has an essential role in metastasis and invasiveness of breast cancer[65]. Reversal of cancer-specific DNA methylation has been pursued using nucleoside-based inhibitors, such as 5-aza-deoxycitidine (also known as decitabine), which is currently evaluated in Phase II clinical trials for myeloid malignancies[66]. Decitabine directly incorporates into the DNA and traps methyl-binding proteins (MBPs) on the DNA template, thereby depleting the cellular store of MBPs. Concomitantly, genomic DNA is demethylated during continuous rounds of DNA replication. Decitabine shows single-agent activity by induction of growth arrest that is followed by mitochondrial apoptosis[67], but is associated with substantial toxicity.

Apoptosis by histone deacetylase inhibitors (HDACi)

Histone acetylation is another important epigenetic regulator of transcription, with histone acetylation being associated with active genes and deacetylation with transcriptionally inactive genes[68]. In cancer, the balance between histone acetylation and deacetylation is often disturbed by inactivating mutations in histone acetyltransferases (HATs) or by overexpression of histone deacetylases (HDACs). HDAC inhibitors (HDACi) such as valproic acid (VPA) showed promising results in clinical trials[69]. *In vitro*, HDACi induce growth arrest, differentiation and apoptosis in various cancer cells[70]. Recent insight into the mode of action of HDACi has revealed that the anti-leukemia activity relies on upregulation of TRAIL and subsequent induction of apoptosis[71,72]. These findings provide an additional rationale for the observed synergistic pro-apoptotic effect of combined treatment with HDACi and TRAIL[73]. Notably, it has become clear that HDACi also modulate acetylation of various non-histone proteins. An interesting example is HSP90, which is destabilized by acetylation, resulting in enhanced proteasomal degradation of several oncogenic client proteins including RAF, ERBB1 and ERBB2[74]. Taken together, as more insight is gained into

the molecular mechanisms of the activity of HDACi, rational combination strategies have been and will be identified. In this respect, combination with inhibition of DNA methylation might further help to reactivate a set of genes that resensitize cells to apoptosis[75].

Conclusions and perspectives

Targeted therapies that are designed to induce apoptosis selectively in cancer cells are currently the most promising anti-cancer strategies. These strategies aim to target and specifically kill tumor cells with no or minimal collateral damage. However, a fundamental problem is still that 'primitive' targeting is often simply not specific enough to enable the delivery of highly toxic agents. Therefore, the problem of cancer selectivity remains an important issue[76–78]. As a consequence, the toxicity of targeted agents has to be reduced often to the point of not being of sufficient therapeutic benefit. It is therefore useful to integrate the concept of how the immune system deals with the targeted delivery of its potentially highly dangerous effector mechanisms. In the immune system, effector mechanisms are tightly controlled both spatially and temporally. An elaborate system of consecutive proof-reading steps and highly selective receptors on specialized effector cells enables the delivery of a highly cytotoxic freight that is activated only at the site of the lesion. In many cases, the immune system eradicates cancerous cells by targeted apoptosis induction with cell-surface-expressed FASL and TRAIL as important effector molecules.

One of the main challenges to be addressed in contemporary targeted therapy is to create technologically more advanced pro-apoptotic molecules. We and others have provided proof of principle for target-cell-restricted apoptosis induction using recombinant fusion proteins in which a tumor-selective antibody fragment is fused to either sTRAIL or sFASL[21–27]. Only upon selective binding to the tumor cell surface the otherwise inactive fusion protein is activated and, subsequently, tumor cell apoptosis is induced in an autocrine or paracrine manner.

Recently, this strategy has been greatly advanced by the construction of a so-called TNF prodrug[79]. The TNF prodrug is a tripartite fusion between a tumor-selective antibody fragment, soluble TNF and a TNF receptor-derived inhibitor module. Additionally, recognition motifs of the matrix metalloproteinase (MMP)-2 were engineered between the TNF and the TNF-receptor-1 domain. After tumor selective binding of the TNF prodrug, the inhibitor module is removed by target cell-expressed MMPs, ensuring strictly antigen-dependent activation of apoptosis.

However, even advanced concepts such as the TNF-prodrug strategy will fail when the targeted tumor cells are resistant to apoptosis due to one or more defects in death receptor or caspase apoptosis pathways. Therefore, reagents that show caspase-independent pro-apoptotic activity, such as Galectin-1, are of particular interest. Alternatively, seemingly

apoptosis-resistant tumor cells can have elevated apoptotic thresholds that cannot be reached without first sensitizing tumor cells using one or more activating drugs. Indeed, the combinatorial use of various pro-apoptotic agents is also of interest. In this respect, the specific targeting of cancer-related anti-apoptotic aberrations, such as silencing of p53 and upregulation of BCL-2 and IAPs, are promising targets for intervention. Because it has recently become clear that cancer cells rely heavily on these aberrations for survival and are in fact more prone to undergo apoptosis than normal cells, modulation of these specific aberrations should provide cancer specificity to some extent. Integration of various concepts can be used to design combinatorial-treatment strategies that enhance or restore the sensitivity of tumor cells to (targeted) apoptosis induction with minimal effects on normal cells. In this respect, the most-promising combinations might involve those drugs that work along different or complementary apoptotic signaling routes with non-overlapping toxicities towards normal cells.

However, an important issue to be addressed is the question of how 'selective' cancer-cell selective is and ultimately can be. Because both normal and cancer cells crucially rely on apoptosis, it is important to consider whether specific modulation of apoptosis in cancer cells is feasible. In other words, is there a large enough therapeutic window between sensitivity to apoptosis in normal and cancer cells? Single-agent therapy is likely to prove not selective enough in most cases. The best way forward seems the combined treatment of cancer cells with therapeutics that are designed to exploit several cancer related aberrations whereby the therapeutic window is increased.

However, the application of such rational combinatorial strategies will rely heavily on the identification of specific cancer-related aberrancies in each patient. Therefore, further development of reliable, cost-effective and high throughput diagnostic tools will be required to enable the successful application of such patient-tailored therapeutic approaches.

Another important issue to address is the occurrence of drug-resistance upon highly selective cancer therapy, such as for the inhibition of the constitutively active breakpointcluster-region (BCR)–ableson-proto-oncogene (ABL) tyrosine kinase in certain forms of leukemia[80]. These findings indicate that even 'magic bullets' seem to lose their magic as single agents.

In addition, all of the approaches discussed here still have to deal with some inherent problems of the respective strategy. For example, the technology for antisense strategies is still fraught with technical limitations, whereas for protein-based therapies immunogenicity is an important issue to address. Humanizing strategies and epitope remodeling are some of the possible ways to reduce immunogenicity. An additional fundamental problem for protein-based therapies in solid tumors is the limited tumor penetration. In such cases, initial tumor-debulking by surgical resection followed by protein-based therapy might prove the best way forward. Taken together, several important questions remain to be

Box 1. Outstanding questions

Despite the elucidation of many of the molecular aberrations that underlie carcinogenesis and the resultant development of a plethora of therapeutic approaches targeting these aberrations, several issues remain to be addressed to determine the feasibility of selective activation of apoptosis in cancer.

• Can apoptosis be selectively activated in cancer cells, being apoptosis a key process for both normal and cancer cells? Although many of the approaches discussed in this article to some extent show selectivity for cancer cells, the 'holy grail' of selective elimination of cancer cells has yet to be uncovered.

• How can adverse effects on other normal tissues be avoided upon application of targeted cancer-cell selective therapeutics?

• How can rational combinatorial strategies be designed to activate divergent or complementary pathways of apoptosis? Can rational combinatorial strategies be identified in order to raise the therapeutic window for cancer-selective activation of apoptosis.

• How can patient-tailored combinatorial strategies be designed efficiently? In other words, how can patients be rapidly and reliably diagnosed?

• How can immunogenicity of protein-based therapeutics be avoided or minimized?

• How can poor tumor-penetration of protein-based therapeutics be overcome?

addressed (Box 1).

Thanks to laser-capture microscopy and DNA-microarray technology, it is now possible to obtain large quantities of gene-expression data from individual cancer cells. However, currently it is still difficult to extract meaningful information from such large quantities of data and to connect them to tumor-specific phenomena or drug information. Nevertheless, further improvements in this field are anticipated that might make it possible to identify hitherto unknown routes for tumor-specific apoptosis induction, which, in turn, can contribute to new discoveries in medical, pharmaceutical and life sciences.

Taken together, as molecular aberrations in apoptosis regulation in cancer cells are elucidated, the rational design of combinatorial approaches paves the way towards enhanced and tumor-selective apoptosis induction that in the future will help fight cancer in a clinical setting.

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Target cell-restricted and -enhanced apoptosis induction by an scFv:sTRAIL fusion protein with specificity for the pancarcinoma-associated antigen EGP2

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chapter 3

Abstract

The apparent tumour selective apoptosis inducing activity of recombinant soluble TRAIL has aroused much interest for use in clinical application. However, to fully exploit its therapeutic potential the characteristics of both the TRAIL receptor system and sTRAIL should be taken into account. Firstly, the wide spread expression of the various TRAIL receptors throughout the human body; secondly, the differential binding affinities and crosslinking requirements of the agonistic receptors TRAIL-R1 and TRAIL-R2; and thirdly, the solution behaviour of particular sTRAIL preparations. Therefore, we constructed a novel TRAIL fusion protein, designated scFvC54:sTRAIL, comprising the human scFv antibody fragment C54 genetically linked to the N-terminus of human soluble TRAIL. The scFvC54:sTRAIL fusion protein was designed to induce apoptosis by crosslinking of agonistic TRAIL receptors only after specific binding of scFvC54:sTRAIL to the abundantly expressed carcinoma-associated cell surface antigen EGP2 (alias EpCAM). Target antigen restricted apoptosis induction was demonstrated for various EGP2-positive tumour cells and could be inhibited by an EGP2 competing antibody. Target antigen binding converted soluble scFvC54:sTRAIL into a membrane bound form of TRAIL that was capable of signalling apoptosis not only through TRAIL-R1, but also through TRAIL-R2. Size-exclusion FPLC indicated that scFvC54:sTRAIL was produced as stable and homogeneous trimers in the absence of detectable TRAIL aggregates. The favourable characteristics of the scFvC54:sTRAIL fusion protein potentially reduce the amount of sTRAIL required for anti-tumour activity and may be of value for the treatment of various human carcinomas.

Introduction

The specific susceptibility of tumour cells to the pro-apoptotic activity of TRAIL, and the apparent lack of susceptibility of normal cells, makes this molecule a promising anti-cancer therapeutic agent. Native TRAIL is expressed as a homotrimeric type II transmembrane protein (memTRAIL), but can also be proteolytically cleaved to form a soluble trimer (sTRAIL)[1,2]. To date, various forms of soluble recombinant TRAIL have been generated, including FLAG-, HIS-, and non-tagged sTRAIL variants, all of which induce apoptosis in a wide range of human tumour cell lines[3]. Potent anti-tumour activity of various sTRAIL variants has been demonstrated in several mouse xenograft models of human cancers, including colorectal cancer[3,4], glioblastoma[4], and breast cancer[5].

Both memTRAIL and sTRAIL can interact with the agonistic TRAIL receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 that initiate apoptosis via their intracellular death domains[6-9]. TRAIL also binds, albeit with lower affinity[10], to TRAIL-R3/DcR1and TRAIL-R4/DcR2[7,11-13], both of which lack a functional death domain. TRAIL-R3 and TRAIL-R4 are considered to act as receptors that potentially modulate TRAIL activity.

Expression of the different TRAIL receptors has been demonstrated not only on various tumours, but also on a wide variety of normal human tissues, indicating that apoptosis induction by TRAIL is a delicately regulated mechanism, much of which is still elusive.

Clustering of TRAIL-R1 and -R2 by TRAIL leads to formation of the Death Inducing Signalling Complex (DISC)[14-17] by recruitment of the adapter protein FADD and resultant binding and activation of initiator caspases-8 and -10[16,18,19-21]. Activated caspase-8 and -10 subsequently activate downstream effector caspases, including caspase-3, -6 and -7, which cleave cytoskeletal and nuclear proteins essential for cell survival such as PARP, alpha-Fodrin, DFF and Lamin A, resulting in apoptosis.

Formation of the TRAIL receptor DISC is strongly enhanced when aggregated or complexed TRAIL binds to TRAIL-R1 or TRAIL-R2. Furthermore, TRAIL-R1 and -R2 were shown to have rather distinct crosslinking requirements for the initiation of apoptosis[22]. Both sTRAIL and memTRAIL can efficiently activate TRAIL-R1 even at low concentrations, whereas TRAIL-R2 can only be activated by memTRAIL or recombinant sTRAIL that is secondarily crosslinked by antibodies. Previously, Wajant et al.[23] demonstrated that signalling capacity of sTRAIL for TRAIL-R2 could be restored by genetic fusion to a recombinant antibody fragment (scFv) recognizing the tumour stroma marker fibroblast activation protein (FAP).

Independently, we developed a versatile expression system in CHO cells for the rapid construction and evaluation of scFv:sTRAIL fusion protein variants. Here we present a detailed characterization of a novel scFv:sTRAIL fusion protein that selectively targets the pancarcinoma-associated membrane antigen EGP2 (also known as GA733-2, EpCAM or CO17-1A antigen). EGP2 is a well-established target antigen that is overexpressed on the cell surface of various human carcinomas such as colorectal, breast, and small cell lung carcinoma[24,25]. In normal epithelia, EGP2 expression is limited to the baso-lateral membrane[26,27]. Furthermore, EGP2 is not shed into the circulation and has been extensively studied in antibody-mediated imaging and immunotherapy of human carcinomas[28-30]. This is the first report on the construction and detailed characterization of a scFv:sTRAIL fusion protein with specificity for the therapeutically relevant pancarcinoma-associated target antigen EGP2.

Materials & Methods

Monoclonal antibodies and scFv antibody fragment

MAb MOC31 is a murine IgG1 with high affinity for human EGP2[31]. The anti-EGP2 scFvC54 has been previously selected from a large semi synthetic phage display library with random human VH-VL pairings and has a VH-(G4S)3-VLκ format[32]. MAb MOC31 and scFvC54 compete for binding to the same epitope on the extracellular domain of EGP2. TRAIL activity neutralizing MAb 2E5 was purchased from Alexis (Kordia Life Sciences, Leiden, The Netherlands). MAb 2E5 neutralizes TRAIL activity by binding to an epitope on the extracellular domain of TRAIL, whereby binding to the various TRAIL receptors is inhibited. A multimeric form of the extracellular domain of EGP2 (sEGP2) was produced and purified as described previously[33]. Multimeric sEGP2 was used to secondarily crosslink scFvC54:TRAIL.

Cell lines

The following cell lines were purchased from the ATCC: A172 and U87MG (both astrocytoma grade IV), SW948 (colorectal adenocarcinoma), Chinese Hamster Ovary (CHO-K1) and Jurkat (human ALL T-cell line). Jurkat cells express high levels of TRAIL-R2 on the cell surface but do not express detectable amounts of TRAIL-R1. As a result, Jurkat cells are only sensitive to crosslinked or aggregated sTRAIL preparations[22,34]. All cell lines were cultured in their respective media supplemented with 10% FCS at 37ºC in humidified 5% CO_2 atmosphere, unless indicated otherwise.

EGP2 transduced cell lines

EGP2 transduced cell lines A172.EGP2, U87MG.EGP2, and Jurkat.EGP2 were generated by infection of the respective parental cell lines with retroviral particles encoding both EGP2 and Enhanced Green Fluorescent Protein (EGFP). In short, EGP2 cDNA was cloned into a retroviral vector derivative of LZRS-pBMN-lacZ[35] kindly provided by Dr. G. Nolan (Stanford University School of Medicine, San Francisco, CA), yielding LZRS-EGP2-IRES-EGFP. To produce retroviral particles, LZRS-EGP2-IRES-EGFP was transfected into the amphotrophic packaging cell line Phoenix, using Fugene-6 transfection reagent according to manufacturer's recommendations (Roche Diagnostics, Almere, The Netherlands). Transfected cells were selected by culturing in the presence of 1 µg/ml puromycin, 300 µg/ml hygromycin, 1 µg/ml diphtheria toxin (BD Biosciences Clontech, Palo Alto, USA). Viral particle-containing supernatant was harvested after 3 days and stored at –80°C until further use. Jurkat, A172 and U87MG cells ($0.5 \cdot 10^6$ cells) were transduced with 1

ml supernatant containing $1 \cdot 10^6$ retroviral particles. Supernatants were removed after overnight incubation, after which cells were transferred to fresh medium. Transduced cells were selected, both for EGFP-fluorescence and EGP2 expression (MAb MOC31-PE), using the MoFlo high-speed cell sorter (Cytomation, Fort Collins, USA).

Analysis of membrane expression of TRAIL-receptors and EGP2

Differential expression of TRAIL-R1, -R2, -R3, and -R4 on the various cell lines was assessed by flow cytometry using TRAIL receptor specific MAbs (Alexis). In short, cells were harvested and washed with serum free medium and resuspended at a concentration of $5 \cdot 10^5$ cells in 100 µl fresh medium containing the respective anti-TRAIL-R MAbs. Specific binding was detected using secondary PE-conjugated antibodies (DakoCytomation, Glostrup, Denmark). EGP2 expression on the tumour cell surface was analyzed by incubation with MOC31-PE. All antibody incubations were carried out for 45 minutes at 0°C and were followed by two washes with serum free medium.

Construction of scFvC54:sTRAIL

Eukaryotic expression plasmid pEE14scFv:sTRAIL was generated for the rapid construction, evaluation, and stable expression of scFv:sTRAIL fusion proteins in CHO-K1 cells. Plasmid pEE14scFv:sTRAIL is based on a vector we described earlier[36]. Important features of this novel vector are the presence of the murine kappa light chain leader peptide encoded upstream of two multiple cloning sites (MCS) that are separated by a 26 residue in-frame linker sequence, and the glutamine synthetase selectable marker gene, which allows for amplified expression of the recombinant protein in the production cell line CHO-K1[37]. The vector exploits the strong CMV promoter to drive recombinant protein expression, while the leader peptide directs the produced fusion protein through the ER and Golgi complex resulting in excretion of fusion protein into the culture supernatant. In the first MCS, a 730 bp DNA fragment encoding scFvC54 derived from phagemid pHENscFvC54, was directionally inserted using the unique *Sfi*I and *Not*I restriction enzyme sites. In the second MCS, a PCR-truncated 593 bp DNA fragment encoding the extracellular domain of human TRAIL (sTRAIL) was cloned in frame using restriction enzymes *Xho*I and *Hin*dIII. TRAIL cDNA truncation was performed by PCR with proofread DNA polymerase according to standard protocol using primers T1: 5'- ATCCTCGAGTCTAGTGGTAGCGGAACCTCTGAG GAAACCATTT-3' (*Xho*I site is underlined) and T2: 5'-CCCAAGCTTCAGGTCAGTTAGCCAAC TAAAAAG-3 (*Hin*dIII site is underlined). Fig.1A depicts a schematic presentation of the monomeric form of the scFvC54:sTRAIL fusion protein.

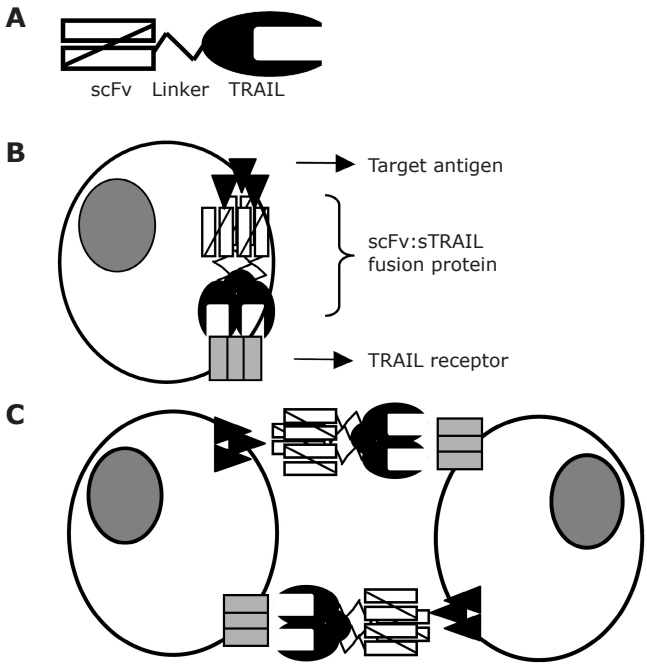

Fig.1. Target cell-restricted triggering of death receptors by scFv:sTRAIL. A; Schematic presentation of a monomeric scFv:sTRAIL fusion protein in which a recombinant antibody fragment (scFv) is genetically fused to human sTRAIL via a linker sequence of 26 amino acid residues. **B;** Target cell-restricted crosslinking of TRAIL receptors by an scFv:sTRAIL fusion protein. In principle, by binding to the target antigen (triangle), scFv:sTRAIL can crosslink agonistic TRAIL receptors and induce apoptosis in a monocellular fashion. **C;** Additionally, scFv:sTRAIL can induce apoptosis in a bicellular fashion in which specific binding to one cell results in the crosslinking of TRAIL receptors on a neighboring tumor cell.

Production of scFvC54:sTRAIL in CHO-K1 cells

CHO-K1 cells were transfected with plasmid pEE14scFvC54:sTRAIL using Fugene 6 reagent (Roche). Stable transfectants were generated by the glutamine synthetase selection method, essentially as described previously[37]. Briefly, pEE14scFvC54:sTRAIL transfected CHO-K1 cells were cultured in GMEM medium (First Link Ltd, West Midlands, UK) supplemented with 5% dialyzed foetal calf serum (Sigma-Aldrich Chemie B.V. Zwijndrecht, The Netherlands) and 100 μM L-methionine-sulfoximine (MSX) (Sigma). Individual clones, obtained after single cell sorting using the Moflo high speed cell sorter, were analyzed for stable and high expression of scFvC54:sTRAIL in the absence of MSX using a solid phase sandwich TRAIL ELISA kit according to manufacturer's recommendations

(Diaclone SAS, Besançon, France). The procedure identified a recombinant CHO-K1 production cell line clone designated 70C1, that stably secreted scFvC54:sTRAIL into the medium at a concentration of 3,44 µg/ml. Large scale production of scFvC54:sTRAIL fusion protein was performed by culturing cell line 70C1 in roller bottles (Greiner Bio-One GmbH, Frickenhausen, Germany) at 37ºC in serum free CHO-S SFM II suspension medium (Gibco, Life Technologies b.v. Breda, The Netherlands) to a density of $5.0 \cdot 10^6$ cells/ml, after which supernatant was harvested (1500xg; 10 min) and stored at -80°C until further use.

Immunoblot analysis of scFvC54:sTRAIL fusion protein

Supernatant derived from scFvC54:sTRAIL production cell line 70C1 was separated by non-reducing SDS/PAGE (12% acrylamide) without sample boiling and was subsequently electroblotted to nitrocellulose (NC). Detection of scFvC54:sTRAIL fusion protein was performed by incubation with anti-TRAIL MAb 2E5 and secondary HRPO-conjugated Goat-anti-Mouse antibody (DAKO), after which specific binding was visualized using chemoluminescence (Roche). Soluble Flag-tagged TRAIL (Alexis) was used as control at a final concentration of 1 µg/ml. All antibody incubations were performed at room temperature in PBS/5% skim milk for 1,5 h and were followed by 3 washes with PBS/0,1%TWEEN.

Size-exclusion FPLC of scFvC54:sTRAIL

The solution behaviour of scFvC54:sTRAIL was analyzed by size exclusion (SE) FPLC using a calibrated HiLoad 16/60 Superdex 200 Prep grade column (Amersham Biosciences AB, Upsala, Sweden) with a bed volume of 120 ml. Five ml supernatant derived from cell line 70C1 was loaded onto the column after which individual samples were collected at 3-minute intervals. All samples were analyzed for their capacity to induce apoptosis using the TRAIL sensitive cell line SW948. Furthermore, all samples were subjected to a sensitive TRAIL specific ELISA to quantitate the scFvC54:sTRAIL content.

Target antigen-restricted cell surface binding of scFvC54:sTRAIL

Target antigen-restricted binding of scFvC54:sTRAIL to the cell surface was assessed by flow cytometry using cell lines SW948 (EGP2-positive) and Jurkat (EGP2-negative). In short, cells were harvested and washed with serum free medium. Subsequently, cells were incubated with scFvC54:sTRAIL (300 ng/ml) in the presence or absence of either the EGP2 competing antibody MOC31 (7,15 µg/ml) or the TRAIL activity neutralizing MAb

2E5 (1 µg/ml). Detection of cell surface bound scFvC54:sTRAIL was performed using anti-TRAIL-PE (Diaclone SAS). All antibody incubations were carried out for 45 min at 0°C and were followed by two washes with serum free medium.

Target antigen-restricted apoptosis induction by scFvC54:sTRAIL

Target antigen-restricted apoptosis induction by scFvC54:sTRAIL (300 ng/ml) was assessed by analysis of the following apoptosis-related cellular phenomena: tumour cell viability, phosphatidyl serine exposure on the outer cell-membrane surface, caspase-8 and -3 activation, DFF degradation by activated caspase-3, and DNA fragmentation. The different analyses were performed in the presence or absence of either MOC31 (7,15 µg/ml) or 2E5 (1 µg/ml) and are described in more detail below.

Viability assay

Tumour cell viability was assessed by MTS assay (Promega Benelux b.v., Leiden, The Netherlands). Briefly, cells were seeded in flat bottom 96-well micro culture plates at a density of $3 \cdot 10^4$ cells/well in 100 µl medium. After overnight culture, spent medium was removed and replaced by 200 µl medium containing the various experimental conditions. After 16 h, MTS assay was performed according to manufacturer's recommendations. Each experimental and control group consisted of six independent wells.

Immunoblot analysis of apoptosis

After treatment of SW948 cells with scFvC54:sTRAIL, intracellular apoptotic features were detected by incubation with antibodies against active caspase-8, active caspase-3 and DFF (PharMingen, San Diego, USA). Briefly, $2.5 \cdot 10^6$ tumour cells were seeded in 6 well plates and treated for 1, 2, 3, 5, 6, 12, and 24 h respectively with the various experimental conditions indicated in Fig.4A and B. Cells were collected by centrifugation (300xg; 10 min), lysed in lysis buffer (20 mM Tris-HCl, 5.0 mM EDTA, 2.0 mM EGTA, 100 mM NaCl, 0,05% SDS, 0,50% NP-40, 1 mM PMSF, 10 µg/ml Aprotein, 10 µg/ml Leupeptin), and sonicated on ice for 2x5 sec. Subsequently, cleared supernatants were collected by centrifugation (15,000xg; 10 min) and separated by SDS-PAGE (12% PAA) under reducing conditions and transferred to NC by electroblotting. Blots were incubated with the appropriate primary monoclonal antibodies and HRPO-conjugated secondary antibody. Specific binding was visualized by chemoluminescence (Roche). All antibody incubations were carried out for 1,5 h in PBS/5% skim milk and were followed by 3 washes with PBS/0,1%Tween.

Flowcytometric analysis of apoptosis induction by scFvC54:sTRAIL

Cells were harvested and resuspended in fresh medium at $1 \cdot 10^6$ cells/ml and incubated for 16 h with the various experimental conditions. After 16 h, cells were harvested by centrifugation (300xg; 5 min.) and analyzed with the AnnexinV-FITC/PI kit (NeXins research, Kattendijke, The Netherlands), the caspscreen flow cytometry apoptosis detection kit (Biovision, Mountain View, USA) and the single strand DNA specific MAb F7-26 (Alexis). AnnexinV-FITC/PI staining was performed according to manufacturer's recommendations and identifies early apoptotic cells with phosphatidyl serine exposure on the outer cell membrane and late apoptotic AnnexinV-FITC/PI double positive cells.

Caspase activation by scFvC54:sTRAIL was analyzed using the Caspscreen flow cytometry apoptosis detection kit that detects cleavage of the non-fluorescent substrate (aspartyl)2-Rhodamine 110 (D2R) into fluorescent Rhodamine 110 by activated caspases.

DNA-fragmentation was analyzed with MAb F7-26 according to manufacturer's recommendations. MAb F7-26 specifically detects apoptotic DNA fragmentation and is based on the high sensitivity of DNA to thermal denaturation in condensed chromatin of apoptotic cells. MAb F7-26 specifically detects deoxycytidine in ssDNA of at least 25-30 bases in length in the absence of any reactivity to double-stranded DNA.

Results

Construction of scFvC54:sTRAIL

DNA encoding the extracellular domain of TRAIL (sTRAIL) was generated by PCR using proofread DNA polymerase. The resulting 593 bp PCR product encoded amino acids 95 - 281 of TRAIL, including cysteine residue number 230 (original position in memTRAIL), which is essential for the structure and activity of TRAIL[38]. The DNA fragment encoding scFvC54 and the sTRAIL PCR product were subsequently inserted in the first and second MCS of eukaryotic expression vector pEE14, yielding plasmid pEE14-scFvC54:sTRAIL. Sequence analysis confirmed the correct and in frame fusion of the scFvC54 and sTRAIL encoding DNAs (data not shown).

Eukaryotic production of scFvC54:sTRAIL

CHO-K1 cells transfected with pEE14-scFvC54:sTRAIL were selected for stable and amplified secretion of scFvC54:sTRAIL using the GS selection method and a TRAIL-specific ELISA. This procedure identified the producer CHO-K1 clone designated 70C1 that secreted 3,44 µg/ml fusion protein into the culture medium. Immunoblot analysis of a 70C1 culture medium sample identified scFvC54:sTRAIL as a fusion protein

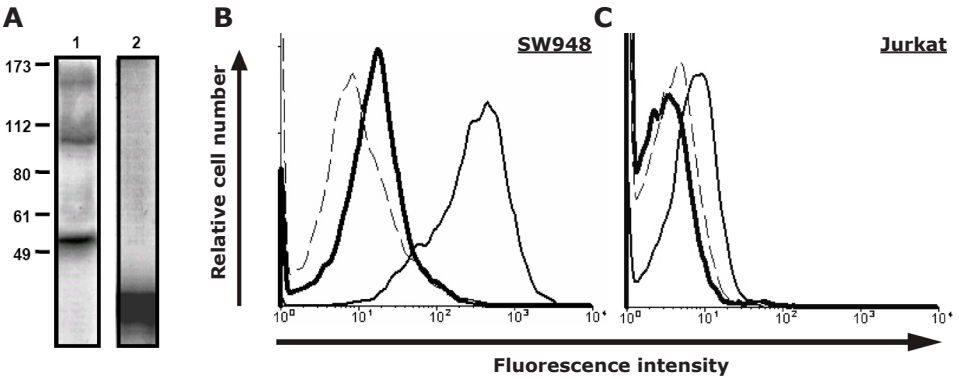

Fig.2. Characterization of scFvC54:sTRAIL. A; Immunoblot analysis of scFvC54:sTRAIL. Lane 1, under non-reducing conditions without sample boiling, scFvC54:sTRAIL was detected as monomers, dimers and trimers (52, 102 and 154 kDa, respectively). Lane 2, Flag-tagged sTRAIL was detected as a monomer of 25 kDa (SDS-PAGE performed under reducing conditions with sample boiling). The relative positions of the molecular weight markers are indicated by dashes. **B;** Flow cytometric analysis of target antigen-specific binding by scFvC54:sTRAIL. scFvC54:sTRAIL shows strong and specific binding to the EGP2-positive tumor cell line SW948 (solid line), which could be specifically inhibited by preincubation with anti-EGP2 MAb MOC31 (dashed line). Unconditioned medium control level is shown as a bold line. **C;** Flow cytometric analysis of scFvC54:sTRAIL binding to target antigen-negative cells. scFvC54:sTRAIL shows minimal binding to EGP2-negative Jurkat cells (solid line), which could be specifically blocked by preincubation with TRAIL-blocking MAb 2E5 (dashed line). Unconditioned medium control level is shown in bold. Binding of scFvC54:sTRAIL to other EGP2-negative cell lines was below detectable levels (data not shown).

with an apparent molecular weight of approximately 52 kDa (Fig.2A, lane 1). This is in close proximity of the molecular weight of 51,180 kDa calculated for monomeric scFvC54:sTRAIL. Flag-tagged sTRAIL was detected as a 25 kDa monomer (Fig.2C, lane 2). The observed molecular weight of scFvC54:sTRAIL is the result of the genetic fusion of scFvC54 (26.6 kDa), 26 amino acid linker (2.6 kDa) and sTRAIL (22 kDa). In addition to monomeric scFvC54:sTRAIL two other bands were detected corresponding to dimeric (102 kDa) and trimeric (154 kDa) forms of scFvC54:sTRAIL. The latter bands were not observed when SDS/PAGE was performed under reducing conditions with sample boiling (data not shown).

Target antigen-restricted binding of scFvC54:sTRAIL

Incubation of SW948 cells (EGP2 pos.) with scFvC54:sTRAIL resulted in strong and specific binding of scFvC54:sTRAIL to the cell surface (Fig.2B, solid line). The observed binding was target antigen specific since pre-incubation with the EGP2 blocking MAb MOC31 completely inhibited scFvC54:sTRAIL binding (Fig.2B, dashed line). Cell surface binding of scFvC54:TRAIL via its TRAIL domain to tumour cells was minimal, as exemplified for

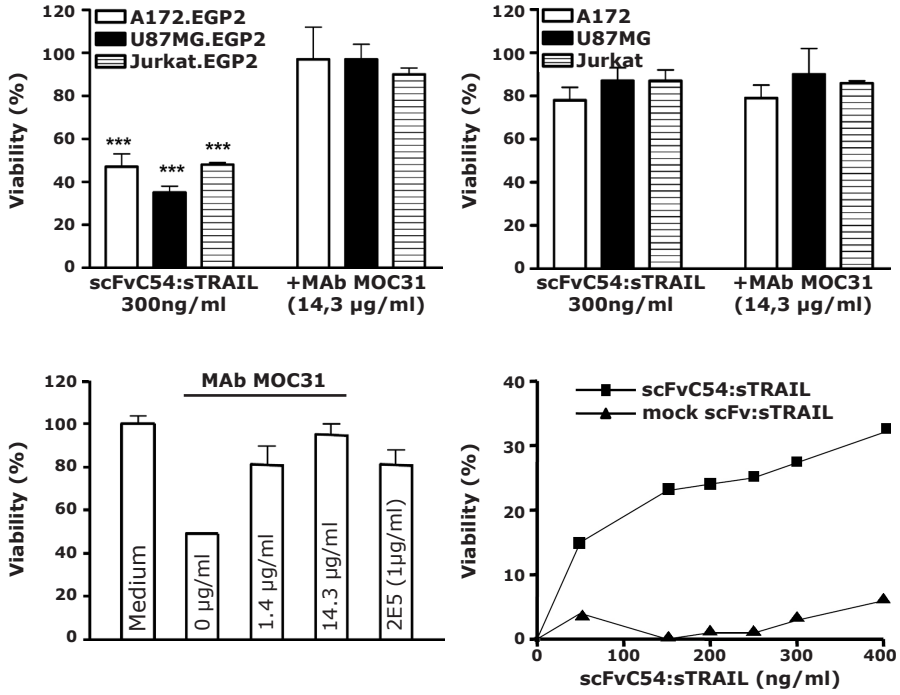

Fig.3. Target antigen-restricted apoptosis induction by scFvC54:sTRAIL. A; Treatment of the EGP2-transduced cell lines A172.EGP2, U87MG.EGP2 and Jurkat.EGP2 with scFvC54:sTRAIL resulted in target antigen-restricted apoptosis induction (measured by MTS assay and indicated by % cell viability), which could be specifically blocked by MAb MOC31 ($p \leq 0.0001$). Mean values and SDs are representatives of 3 independent experiments. **B;** Incubation of the EGP2-negative parental cell lines A172, U87MG and Jurkat with scFvC54:sTRAIL resulted in background levels of apoptosis (measured by MTS assay and indicated by % cell viability), which could not be blocked by preincubation with MAb MOC31. Mean values and SDs are representatives of 3 independent experiments. **C;** Treatment of EGP2-positive SW948 cells with scFvC54:sTRAIL (300 ng/ml) strongly induced apoptosis (measured by MTS assay and indicated by % cell viability), which could be inhibited in a dose-dependent manner by preincubation with increasing amounts of MAb MOC31 (bars 3 and 4). Cell viability in medium without scFvC54:sTRAIL is shown in bar 1. Co-incubation with TRAIL-neutralizing MAb 2E5 abrogated apoptosis induction (bar 5). Incubation with MAb MOC31 or MAb 2E5 alone had no effect on cell viability (data not shown). Mean values and SDs are representatives of 3 independent experiments. **D;** Jurkat.EGP2 treated with increasing concentrations of an scFv:sTRAIL fusion protein containing an scFv of irrelevant target specificity (triangle) only showed background level of apoptosis induction. Treatment with increasing concentrations of scFvC54:sTRAIL (square) resulted in a dose-dependent induction of apoptosis. Parental Jurkat cells were not sensitive to apoptosis induction by scFvC54:sTRAIL (data not shown).

Jurkat cells (EGP2 neg.) to which weak TRAIL domain-mediated binding was observed (Fig.2C, solid line). This binding could be completely inhibited by co-incubation with MAb 2E5 (Fig. 2C, dashed line).

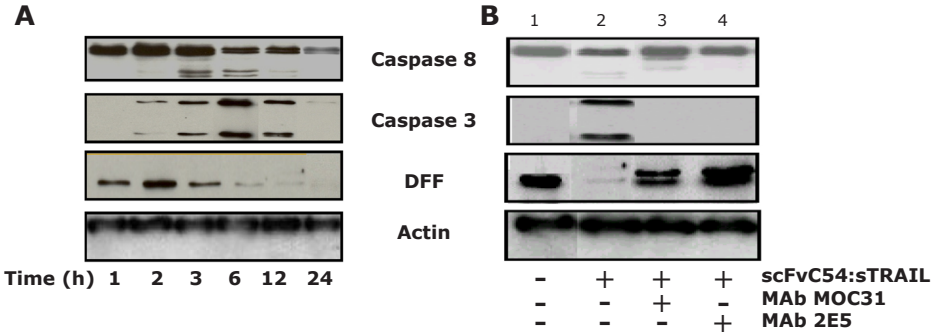

Fig.4. Immunoblot analysis of apoptosis induction by scFvC54:sTRAIL in SW948. A; Activation of initiator caspase-8 (MW 43kDa) and effector caspase-3 (MW 29 kDa) was apparent after three hours and maximum activation levels were reached after 6 hours. Levels decreased after 12 and were below detection after 24 hours. Cleavage of the active caspase-3 target protein DFF was detected after 3 hours by a decrease in intact DFF (MW 45 kD). DFF levels decreased further until below detection after 12 and 24 hours. **B;** Co-incubation of SW948 with scFvC54:sTRAIL and target antigen blocking MAb MOC31 or TRAIL neutralizing MAb 2E5 completely inhibited scFvC54:sTRAIL induced apoptosis (12 hours). After blocking with MAbs MOC31 and 2E5, additional band of approximately 50 kDa appeared due to staining of murine IgG1. For all indicated time points no apoptosis was detected in medium control (data not shown).

Target antigen-restricted apoptosis induction by scFvC54:sTRAIL

Treatment with scFvC54:sTRAIL resulted in prominent apoptosis induction, as measured by a strong reduction of cell viability, in all the EGP2-positive tumour cell lines tested (Fig.3A), whereas viability was only minimally reduced in the EGP2-negative cell lines (Fig.3B). Induction of apoptosis by scFvC54:sTRAIL was target antigen dependent since pre-incubation with saturating amounts of MAb MOC31 restored cell viability to medium control in all EGP2-positive cell lines (Fig.3A). Co-incubation of SW948 cells with a fixed concentration of scFvC54:sTRAIL and increasing concentrations of MAb MOC31 resulted in a dose-dependent reduction in apoptosis (Fig.3C). Similar results were obtained for all other EGP2-positive tumour cell lines (data not shown). Furthermore, incubation of EGP2-positive tumour cells with an scFv:sTRAIL fusion protein containing an scFv of irrelevant target specificity did not induce apoptosis (Fig.3D). Apoptosis induction by scFvC54:sTRAIL was abrogated by co-incubation with TRAIL-neutralizing MAb 2E5 (Fig.3C), which was observed for all cell lines tested (data not shown).

Apoptotic features induced by scFvC54:sTRAIL

Apoptotic features induced by scFvC54:sTRAIL were analyzed at elapsed time points of 1, 2, 3, 6, 12, and 24 h. For the initiator caspase-8 and the effector caspase-3 the

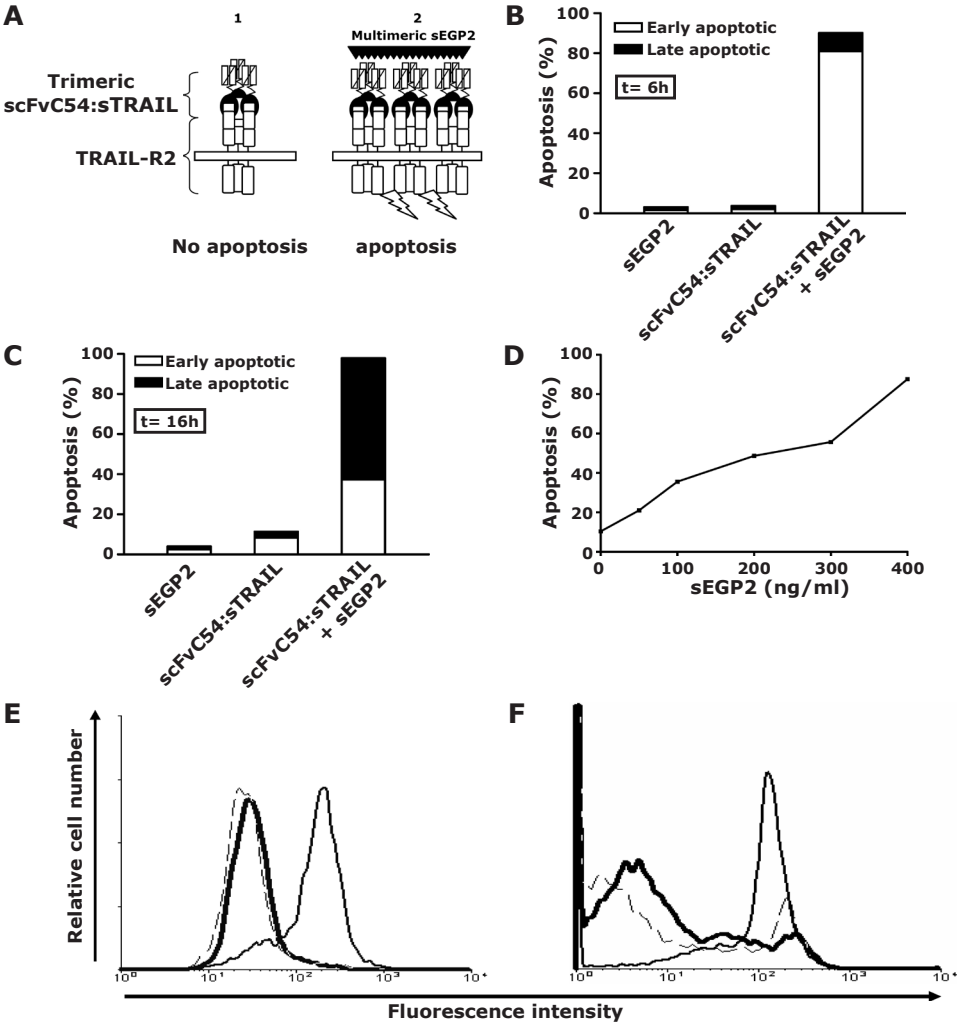

Fig.5. TRAIL-R2 signalling by scFvC54:sTRAIL. A; Schematic representation of treatment of Jurkat cells (EGP2 and TRAIL-R1 negative, TRAIL-R2 positive) with scFvC54:sTRAIL (I) or with scFvC54:sTRAIL crosslinked by a multimeric form of the extracellular domain of EGP2 (sEGP2) (II). **B/C;** Jurkat cells treated with crosslinked scFvC54:sTRAIL showed a marked increase in early (white bars) and late apoptotic cells (black bars) after 6 and 16 hours. Incubation with scFvC54:sTRAIL alone had no effect on apoptosis induction. **D;** A strong positive correlation (r^2=0,96) was found between the amount of multimeric sEGP2 present and the level of apoptosis induction by a fixed concentration of scFvC54:sTRAIL (350 ng/ml). **E;** Incubation with crosslinked scFvC54:sTRAIL induced caspase activation (solid line). Incubation with scFvC54:sTRAIL alone resulted in no caspase activation (dashed line) with levels comparable to medium control (bold line). **F;** Crosslinked scFvC54:sTRAIL induced strong and complete DNA fragmentation after 16 hours (solid line), while in medium control (bold line) and scFvC54:sTRAIL alone (dashed line) no DNA fragmentation was observed

following pattern was observed; activation was detectable after 3 h, which increased up to 6 h (Fig.4A). Activation levels of both caspase-8 and -3 decreased after 12 h. After 24 h no active caspase-8 or -3 could be detected. Increasing levels of active caspase-3 corresponded closely with declining DFF levels after 3 h and 6 h, with no DFF detectable after 12 h and 24 h (Fig.4A). Co-incubation of SW948 cells with scFvC54:sTRAIL and MAb MOC31 or MAb 2E5 completely inhibited apoptosis induction (Fig.4B, lanes 3 and 4, respectively). Treatment with culture medium containing no scFvC54:sTRAIL did not result in any of the apoptotic features analyzed (Fig.4B, lane 1).

TRAIL-R2 activation by scFvC54:sTRAIL

Jurkat cells (EGP2 neg.; TRAIL-R1 neg.; TRAIL-R2 pos.) were used to assess the specific activation of TRAIL-R2 by scFvC54:sTRAIL (Fig.5A). Jurkat cells proved to be insensitive to incubation with scFvC54:sTRAIL even for prolonged periods of time (Fig.5B and C; 6 h, 16 h). However, when Jurkat cells were incubated with scFvC54:sTRAIL in the presence of a multimeric form of the extracellular domain of EGP2 (sEGP2), strong induction of apoptosis was observed (Fig.5B and C; 6 h and 16 h). When increasing amounts of multimeric sEGP2 were added in the presence of a fixed concentration of scFvC54:sTRAIL (350 ng/ml), apoptosis was induced in a dose-dependent manner (Fig.5D). Jurkat cells cultured in the presence of the highest concentration multimeric sEGP2 alone showed no signs of apoptosis (data not shown). Furthermore, activation of caspase-3 (Fig.5E) and complete DNA fragmentation (Fig.5F) was only detected when cells were treated with scFvC54:sTRAIL secondarily crosslinked with sEGP2.

Solution behaviour of scFvC54:sTRAIL

Supernatant containing scFvC54:sTRAIL was subjected to size exclusion (SE) FPLC. As indicated in Fig.6A, apoptosis induction of the TRAIL sensitive cell line SW948 was restricted to the individual samples collected after 95 to 115 minutes. TRAIL ELISA subsequently confirmed that only these fractions contained scFvC54:sTRAIL (Fig.6B). This elution peak of scFvC54:sTRAIL corresponded to a MW of approximately 160 kDa, which is in close proximity of the 154 kDa calculated for trimeric scFvC54:sTRAIL.

Discussion

Human sTRAIL appears to be a promising new anti-cancer agent. However, the widespread expression of TRAIL receptors throughout the human body and the recently reported possible TRAIL-related toxicity towards certain normal cells, at least of certain

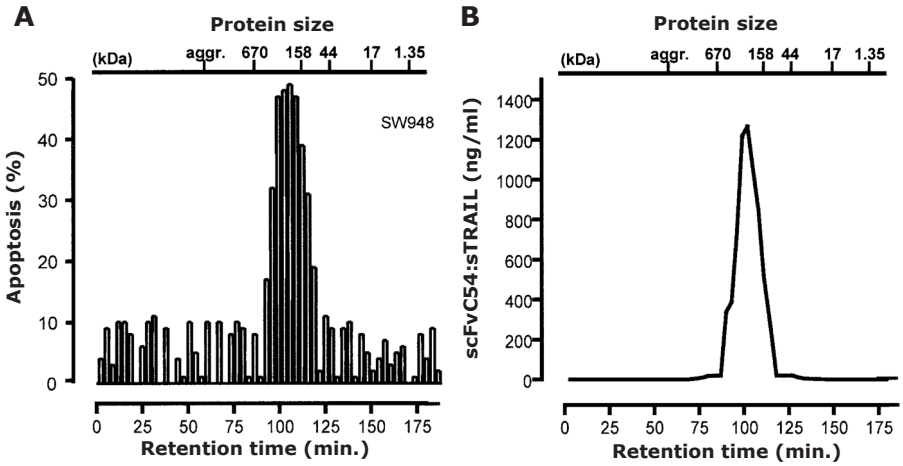

Fig.6. Solution behavior of scFvC54:sTRAIL analyzed by size exclusion FPLC. A; apoptosis induction by individual s.e.FPLC samples on the TRAIL sensitive cell line SW948. **B;** Concentration of scFvC54:sTRAIL in individual s.e.FPLC samples. The retention time of the calibration standards are indicated in the figures. Aggregates, 58 min; Thyroglobulin (bovine), 670kD, 85 min; γ-globulin (bovine), 158kD, 106 min; Ovalbumin (chicken), 44kD, 124 min; Myoglobin (horse), 17kD, 138 min; Vitamin B-12, 1.35kD, 161 min.

recombinant forms of this cytokine, might hamper its clinical development. Augmentation of the therapeutic value of sTRAIL can be achieved by increasing its tumour selective binding properties through the genetic fusion to a tumour-selective antibody fragment[23]. Here we demonstrated that specific targeting of sTRAIL to EGP2-positive cancer cells can be attained by the scFvC54:sTRAIL fusion protein, in which the carcinoma specific antibody fragment scFvC54 is genetically linked to the N-terminus of human sTRAIL. The high affinity scFvC54 domain specifically recognizes EGP2, an established cell surface target antigen that is highly overexpressed on a variety of human carcinomas[39-41]. Specific binding of scFvC54:sTRAIL to EGP2-positive tumour cells was readily demonstrated by flow cytometry. Pre-incubation with competing anti-EGP2 MAb MOC31 selectively blocked the binding in a dose-dependent manner. Binding to EGP2-negative cell lines was below detectable levels, except for Jurkat cells to which a weak extracellular binding was observed that could be blocked by a TRAIL-neutralizing MAb. Together this demonstrated that the scFvC54 targeting domain strongly enhanced the tumour-selective binding of scFvC54:sTRAIL to EGP2-positive tumour cells only.

FACS data further indicated that EGP2-specific binding converted soluble scFvC54:sTRAIL into an artificial membrane bound form of TRAIL. Since the number of EGP2 target molecules greatly exceeded that of the TRAIL-receptors on the same cell, a surplus of

sTRAIL domains was available for subsequent crosslinking of agonistic TRAIL receptors on neighbouring tumour cells. When EGP2-positive tumour cells (Jurkat.EGP2, A172.EGP2, and U87MG.EGP2) were subjected to treatment with scFvC54:sTRAIL, an efficient induction of apoptosis was observed, which in all cases could be inhibited by pre-incubation with MAb MOC31 or a TRAIL-neutralizing MAb. This indicated that scFvC54:sTRAIL efficiently induced bi- or multi-cellular reciprocal fratricide apoptosis in a target antigen-restricted fashion. This principle has been previously described in a study on the specific targeting of FAS-mediated apoptosis induction[42]. In this study by Jung et al., a bi-specific antibody fragment was used comprising a non-activating monomeric CD95 antibody hybridized to a second monomeric antibody targeting CD20 present on the same tumour cell.

Recently, it was shown that TRAIL receptors 1 and 2 have quite distinct crosslinking requirements for the initiation of apoptosis[22]. TRAIL-R2 appears to signal apoptosis only after efficient receptor crosslinking by either native memTRAIL, aggregated sTRAIL variants, or by sTRAIL preparations secondarily crosslinked by antibodies. Apoptosis signalling by TRAIL-R1 appears to be relatively independent of the receptor crosslinking characteristics of a particular form of sTRAIL. Furthermore, it was shown that TRAIL-R2 had superior binding affinity for TRAIL, resulting in predominant binding of sTRAIL to TRAIL-R2 over TRAIL-R1[10].

To analyze the TRAIL receptor crosslinking effects of scFvC54:sTRAIL, we exploited Jurkat cells, that express TRAIL-R2 but no detectable levels of TRAIL-R1. Jurkat cells are resistant to relatively high concentrations of non-aggregated sTRAIL. When Jurkat. EGP2 transfectant cells were subjected to scFvC54:sTRAIL treatment, efficient induction of apoptosis of up to 60% was achieved, indicating that target antigen-restricted apoptosis induction in these cells is initiated via TRAIL-R2 crosslinking. Furthermore, when parental Jurkat cells were subjected to a fixed concentration of scFvC54:sTRAIL, in the presence of increasing amounts of a multimeric form of sEGP2, a dramatic increase in apoptosis induction was observed, which directly correlated to the concentration of crosslinking multimeric sEGP2 added (see Fig.5D). From this it can be concluded that the target antigen-restricted apoptosis-inducing capacity of scFvC54:sTRAIL is directly proportional to the degree of TRAIL-R2 crosslinking. Moreover, this data indicates that scFvC54:sTRAIL can overcome TRAIL-resistance related to the differential expression of TRAIL-R2 over TRAIL-R1 as is observed for many different TRAIL resistant cell lines. The parental cell lines Jurkat, A172 and U87MG, used in the present study, exemplify the preferential expression of TRAIL-R2 over TRAIL-R1 and the subsequent insensitivity to treatment with scFvC54:sTRAIL. Interestingly, SW948 cells (EGP2-positive colon

carcinoma cells) expressing both TRAIL-R1 and TRAIL-R2, were almost completely rescued from apoptosis by pre-incubation with blocking MAb MOC31 (Fig.3C). This implies that, even in the presence of TRAIL-R1, scFvC54:sTRAIL-mediated apoptosis appears to be predominantly initiated by TRAIL-R2. Although not formally proven here, it appears that this possibly is the result of the preferential crosslinking capacity of scFvC54:sTRAIL for the high affinity TRAIL-R2 receptor over TRAIL-R1, which binds TRAIL with lower affinity[10].

The current data independently corroborate with the results previously published by Wajant et al.[23]. Wajant et al demonstrated that the restricted signaling capacity of sTRAIL could be converted into a TRAIL-R2 stimulating ligand after genetic fusion to a scFv antibody fragment specific for the tumour stroma marker Fibroblast Activation Protein (FAP).

Recently, several papers reported on the apoptosis-inducing potential of certain recombinant sTRAIL preparations towards primary human cells such as normal human hepatocytes[43], keratinocytes[44], prostrate epithelial cells[45], and brain tissue[46]. It has been suggested that this potential toxicity is related to high molecular weight sTRAIL aggregates present in certain sTRAIL preparations[47]. Purified his-tagged sTRAIL, refolded from bacterial expression systems, contained TRAIL-aggregates that might be directly responsible for the hepatocyte toxicity observed for this particular preparation. Thus, the production of non-aggregated sTRAIL derivatives appears to be important in order to avoid organ-specific or systemic toxicity.

In the present study, we aimed at producing biologically active and correctly folded scFvC54:sTRAIL by directing it through the endoplasmatic reticulum of eukaryotic CHO-K1 cells, thus taking advantage of the associated stringent quality control mechanisms ensuring that only correctly folded and non-aggregated fusion protein is secreted into the culture medium. The anti-FAP scFv:sTRAIL fusion protein, described by Wajant et al[23], was transiently expressed from transfected COS-7 cells. However, transient expression systems are intrinsically subject to batch-to-batch variations and in their study no data was presented on molecular weight, or solution behaviour of the produced fusion protein.

Anticipating its clinical potential, we produced scFvC54:sTRAIL in Chinese Hamster Ovary (CHO) cells, a currently favoured host cell type for the production of therapeutic recombinant proteins. The Glutamine Synthetase system was used to generate a CHO production cell line clone 70C1 that stably secreted high levels of scFvC54:sTRAIL in to the medium and allowed for large scale production. To assess for the presence of scFvC54:sTRAIL aggregates in the production medium, we exploited the high sensitivity of

Jurkat cells to such aggregates. When Jurkat cells were exposed to prolonged incubation with medium containing up to 3,4 µg/ml scFvC54:sTRAIL, no signs of apoptosis or toxicity were observed.

Subsequent analysis of the solution behaviour of scFvC54:sTRAIL by size exclusion (SE) FPLC produced a single TRAIL activity peak corresponding to a molecular weight of approximately 160 kDa (Fig.6). This is in close proximity of the 154 kDa that can be calculated for scFvC54:sTRAIL trimers. From this it can be concluded that scFvC54:sTRAIL is produced as soluble homogeneous trimers with no or only minimal aggregate formation.

Stable trimeric scFvC54:sTRAIL contains three identical scFvC54 domains, which potentially enhances binding to EGP2-positive cells by the associated avidity effect. Enhanced avidity has been shown to be beneficial for in vivo tumour targeting in many antibody-based therapeutic strategies[48,49]. In our experiments, equimolar concentrations of the bivalent high affinity (<10nm) anti-EGP2 MAb MOC31 were needed to inhibit binding of trimeric scFvC54:sTRAIL to EGP2-positive cells, indicating that both molecules are at least of similar affinity. We designed scFvC54:sTRAIL to contain a spacer of 26 amino acids between the scFvC54 domain and sTRAIL. The length and flexibility of this spacer, containing an SSGSG hydrophilic region, were employed to prevent steric hindrance during simultaneous binding of trimeric scFvC54:sTRAIL to three EGP2 molecules and one trimeric TRAIL-R molecule.

In conclusion, to fully exploit the therapeutic potential of sTRAIL, the characteristics of both the TRAIL receptor system and sTRAIL should be taken into account. Firstly, the wide-spread expression of the various TRAIL receptors throughout the human body; secondly, the differential binding affinities and crosslinking requirements of the agonistic receptors TRAIL-R1 and TRAIL-R2; and thirdly, the solution behaviour of particular sTRAIL preparations. The fusion protein scFvC54:sTRAIL complies with these notions and is the first example of a sTRAIL variant with enhanced tumour selective apoptosis induction towards EGP2-positive tumour cells. The favourable characteristics of scFvC54:sTRAIL potentially reduce the amount of TRAIL required for anti-tumour activity and may thereby reduce the risk of potential toxicity associated with conventional non-targeting sTRAIL preparations.

Nevertheless, it cannot be completely excluded that even scFv:sTRAIL fusions proteins such as scFvC54:sTRAIL, described here, and the anti-FAP scFv:sTRAIL fusion protein, described by Wajant et al, might exert certain toxic effects towards normal human tissues expressing the respective antigen. In normal epithelia, EGP2 is shielded by an intact

basal membrane[26,27], thereby reducing the risk of targeting scFvC54:sTRAIL to non-malignant epithelial tissue. Toxicity of scFvC54:sTRAIL can, in part, be analyzed with the human EGP2 transgenic mouse[50] model that was recently generated in our lab. In this transgenic mouse, human EGP2 expression exhibits an authentic epithelial expression pattern that is comparable to the human situation. Unfortunately, human FAP-transgenic animal models are currently not available, which might potentially hamper the further pre-clinical evaluation of the anti-FAP scFv:sTRAIL fusion protein.

From the present *in vitro* study we conclude that targeting of sTRAIL to the abundant tumour-associated target antigen EGP2, and possibly to a number of other known surface-expressed tumour antigens like the Epidermal Growth Factor Receptor, is a promising anti-tumour strategy that might be useful in clinical application for various human carcinomas.

Acknowledgements

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chapter 4

Exceptionally potent anti-tumour bystander activity of an scFv:sTRAIL fusion protein with specificity for EGP2 towards target antigen-negative tumour cells.

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Abstract

Previously, we reported on the target cell-restricted fratricide apoptotic activity of scFvC54:sTRAIL, a fusion protein comprising human soluble TRAIL genetically linked to the antibody fragment scFvC54 specific for the cell surface target antigen EGP2. In the present study, we report that the selective binding of scFvC54:sTRAIL to EGP2-positive target cells conveys an exceptionally potent pro-apoptotic effect towards neighbouring tumour cells that are devoid of EGP2 expression (bystander cells). The anti-tumour bystander activity of scFvC54:sTRAIL was detectable at target- to bystander cell ratios as low as 1:100. Treatment in the presence of EGP2-blocking or TRAIL-neutralizing antibody strongly inhibited apoptosis in both target and bystander tumour cells. In the absence of target cells, bystander cell apoptosis induction was abrogated. The bystander apoptotic activity of scFvC54:sTRAIL did not require internalization, enzymatic conversion, diffusion, or communication between target and bystander cells. Furthermore, scFvC54:sTRAIL showed no detectable signs of innocent bystander activity towards freshly isolated blood cells. Further development of this new principle is warranted for approaches where cancer cells can escape from antibody-based therapy due to partial loss of target antigen expression.

Introduction

In recent years several antibody-based therapies that target tumour-associated membrane antigens have entered clinical trials with promising results[1-3]. However, curative treatment is frequently not achieved due to therapy-resistant recurrences emerging after initial rounds of seemingly successful treatment[4-6]. It has been shown that within one tumour mass different stages of malignant progression and various oncogenic mutations can occur simultaneously, leading to the development of heterogeneous tumour cell phenotypes[7-12]. Heterogeneous and lost target antigen expression are likely to be responsible for many of the therapeutic failures observed in current antibody-based therapies[4-6].

Therefore, strategies have been developed to take advantage of the so-called 'bystander effect', which aims to eliminate tumour cells with reduced or lost target antigen expression. The bystander effect is based on the principle that targeted tumour cells are not only eliminated, but are also exploited to convey the therapeutic effect towards neighbouring tumour cells devoid of target antigen expression. Bystander effects have been described for several antibody-based therapeutic approaches[13,14], and more recently

for gene therapy using FASL and TRAIL, two members of the Tumour Necrosis Family of death inducing ligands[15-18].

TRAIL is of particular interest for its tumour-restricted apoptosis inducing capacity in a wide range of neoplastic cells while sparing normal tissues. TRAIL is expressed as a type II transmembrane protein (memTRAIL)[19,20] on a broad spectrum of tissues ranging from peripheral blood lymphocytes, spleen, and thymocytes to many solid organs but is absent in brain, liver, and testis.

A unique TRAIL receptor system has been uncovered in which the distinct receptors TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, and osteoprotegerin (OPG), can differentially bind and interact with TRAIL. After ligation, TRAIL-R1 and TRAIL-R2 recruit the intracellular FAS-associated Death Domain adapter protein (FADD) and the initiator caspase-8 or -10, thereby forming the Death Inducing Signalling Complex (DISC)[21-28]. Assembly of the DISC results in activation of caspase-8 or -10 that subsequently cleave and activate effector caspases, such as caspase-3, -6, and -7, leading to e.g. PARP cleavage and ultimately apoptotic cell death. TRAIL-R3, TRAIL-R4, and OPG lack (functional) death domains and after ligation, do not induce apoptosis.

TRAIL-R1 and TRAIL-R2 have a broad and partly overlapping pattern of expression, suggesting that they may serve as an alternate or 'backup' system, allowing the immune system to control aberrant cells even if one of the receptors has failed. Recently, it was shown that TRAIL-R1 and TRAIL-R2 have rather distinct crosslinking requirements for the initiation of apoptosis[29]. Both recombinant soluble TRAIL (sTRAIL), consisting of the extra-cellular domain of TRAIL, and memTRAIL can efficiently activate TRAIL-R1 even at low concentrations, whereas TRAIL-R2 can only be activated by memTRAIL or sTRAIL that is secondarily crosslinked by antibodies.

To date, various forms of recombinant soluble TRAIL have been generated of which potent anti-tumour activity has been demonstrated in several xenograft mouse models of human cancers, including colorectal cancer[30,31], glioblastoma[31], and breast cancer[32]. These sTRAIL preparations have retained the selective apoptotic activity towards transformed cells, but lack an intrinsic targeting capacity that allows for preferential binding to TRAIL receptors expressed on tumour cells. Moreover, sTRAIL is not very effective in signalling apoptosis in tumour cells that predominantly express TRAIL-R2.

It has been shown that crosslinking of agonistic TRAIL receptors is required to efficiently obtain cell death. In a previous report, Wajant et al.[33] demonstrated that signalling capacity of sTRAIL for TRAIL-R2 could be restored by genetic fusion to a recombinant antibody fragment (scFv) recognizing the tumour stroma marker fibroblast activation protein

(FAP). Independently, we developed a TRAIL fusion protein, designated scFvC54:sTRAIL, in which the human scFv antibody fragment C54 is genetically linked to the N-terminus of human soluble TRAIL[34]. The high affinity scFvC54 antibody domain specifically targets EGP2 (also known as epithelial cell adhesion molecule (Ep-CAM), or CO17-1a antigen), an established cell surface target antigen over-expressed on a variety of carcinomas. Selective binding to EGP2 results in accretion of scFvC54:sTRAIL at the cell surface of targeted cells only, converting soluble scFvC54:sTRAIL into a membrane bound form of TRAIL. Subsequently, a surplus of sTRAIL domains displayed on the target cell surface is available for the crosslinking of TRAIL-R2 on neighbouring tumour cells, resulting in efficient and target antigen-restricted reciprocal fratricide apoptosis induction.

In the present study, we analyzed whether targeting of scFvC54:sTRAIL to EGP2-positive cells can be used to convey a pro-apoptotic bystander effect towards neighbouring tumour cells devoid of EGP2 expression, as schematically depicted in Fig.1. Interestingly, we observed an exceptionally potent bystander apoptotic effect of scFvC54:sTRAIL, which critically depended on the presence of EGP2-positive target cells. Bystander apoptosis induction by scFvC54:sTRAIL might be applicable for the treatment of human cancer cells that escape current antibody-based therapy due to partial loss of target antigen expression.

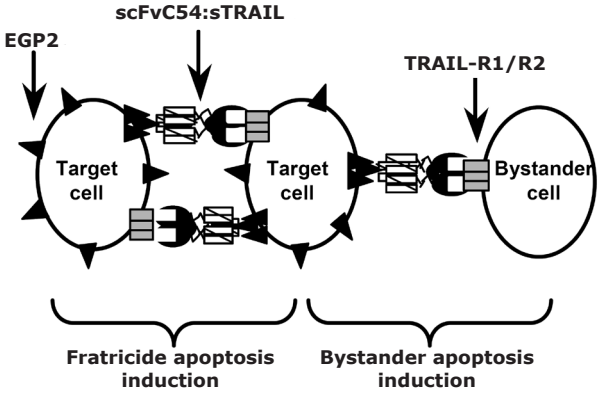

Fig.1. Target cell and bystander cell apoptosis induction by scFvC54:sTRAIL. Binding of scFvC54:sTRAIL to the abundantly expressed target antigen EGP2 (▲) results in immobilization of scFvC54:sTRAIL at the cell surface of EGP2-positive cells only. Subsequently, membrane bound scFvC54:sTRAIL induces fratricide apoptosis by reciprocal crosslinking of TRAIL-R1/-R2 (▭) on neighboring EGP2-positive target cells. Analogously, immobilized scFvC54:sTRAIL on target cells can induce crosslinking of agonistic TRAIL receptors on the cell surface of a neighboring tumor cell devoid of EGP2 expression, resulting in apoptosis induction of one or more bystander cells (diagram is not to scale).

Materials & Methods

Monoclonal antibodies and scFv antibody fragment

Monoclonal antibody (MAb) MOC31 is a murine IgG1 with high affinity specificity for human EGP2[35]. MAb MOC31 was directly labelled with phyco-erythrin (PE), yielding MOC31-PE using standard procedures. The anti-EGP2 scFvC54 (kindly provided by Prof. T. Logtenberg, Utrecht University, The Netherlands), has been previously selected from a large semi-synthetic phage display library with random human VH-VL pairings and has a VH(G4S)3-VL format[36]. MAb MOC31 and scFvC54 compete for binding to the same epitope on the extracellular domain of EGP2. A multimeric form of the extracellular domain of EGP2 (sEGP2) was produced and purified as described previously[37]. Where indicated, multimeric sEGP2 was used to secondarily crosslink scFvC54:sTRAIL. TRAIL-neutralizing MAb 2E5 was purchased from Alexis (Kordia Life Sciences, Leiden, The Netherlands). MAb 2E5 neutralizes TRAIL activity by binding to an epitope on the extracellular domain of TRAIL that inhibits binding to the various TRAIL receptors.

Cell lines and EGP2 transfectants

Human cell lines Jurkat (acute lymphoblastic T cell leukemia), Ramos (B cell lymphoma), and U87MG (glioblastoma), all of which are EGP2-negative, were purchased from the ATCC. EGP2-positive variants of the above cell lines were generated by retroviral transduction. In short, EGP2 cDNA was cloned into a retroviral vector derivative of LZRS-pBMN-lacZ[38] (kindly provided by Dr. G. Nolan, Stanford University School of Medicine, San Francisco, USA), yielding LZRS-EGP2-IRES-EGFP. To produce retroviral particles, LZRS-EGP2-IRES-EGFP was transfected into the amphotrophic packaging cell line Phoenix, using Fugene-6 transfection reagent according to manufacturer's recommendations (Roche Diagnostics, Almere, The Netherlands). Transfected cells were selected by culturing in the presence of 1 μg/ml puromycin, 300 μg/ml hygromycin, and 1 μg/ml diphtheria toxin (BD Biosciences Clontech, Palo Alto, USA). Viral particle-containing supernatant was harvested after 3 days and used to transduce Jurkat, Ramos and U87MG cells. After overnight incubation, viral particle-containing supernatant was replaced by fresh medium. Transduced cells were subsequently sorted for simultaneous EGFP-fluorescence and EGP2 expression as detected by MOC31-PE using the MoFlo high-speed cell sorter (Cytomation, Fort Collins, USA). Analogous methods were employed to generate c-FLIP$_L$ encoding retroviral particles, which were used to transduce Ramos cells. Ectopic over-expression of c-FLIP$_L$ in Ramos.c-FLIP$_L$ transduced cells was confirmed by immunoblotting of intracellular protein extracts. All cell lines were cultured at 37ºC in humidified 5% CO_2 atmosphere.

Suspension cell lines (Jurkat, Jurkat.EGP2, Ramos, Ramos.EGP2, Ramos.c-FLIP$_L$) were cultured in RPMI (Cambrex, New Jersey, USA) supplemented with 15% foetal calf serum (FCS). Adherent cell lines (U87MG, U87MG.EGP2) were cultured in DMEM (Cambrex) supplemented with 10% FCS.

Expression of TRAIL receptors

Membrane expression levels of TRAIL-receptors 1, 2, 3, and 4, were analyzed by flow cytometry using a TRAIL-receptor antibody kit purchased from Alexis. Briefly, cells were harvested, washed using serum free RPMI, and resuspended in 100 μl fresh medium containing the appropriate primary MAb. Specific binding of the primary antibody was detected using a PE-conjugated secondary antibody (DAKO, Glostrup, Denmark). All antibody incubations were performed at 0°C for 45 min and were followed by two washes with serum free medium.

Production of scFvC54:sTRAIL

The fusion protein scFvC54:sTRAIL, comprising the scFvC54 targeting domain, an intra-chain linker, and the sTRAIL effector domain, was produced in Chinese Hamster Ovary (CHO-K1) cells as previously described[34]. Briefly, the expression plasmid pEE14scFvC54:sTRAIL was transfected to CHO-K1 cells, after which cells were selected for amplified medium secretion of the fusion protein using the glutamine synthetase method as described before[39]. Single cell sorting of transfectants using the MoFlo high speed cell sorter (Cytomation) identified CHO-K1 clone 70C1 that stably secreted 3.44 μg/ml scFvC54:sTRAIL into the culture medium. Using the same procedures, Mock-scFvH22:sTRAIL, directed at the antigen CD64, not present on the cell lines used in this study, was generated and added in experiments where indicated.

Apoptosis induction assessed by viability assay

Where indicated, apoptosis induction apparent from loss of tumour cell viability was assessed by MTS assay (Promega Benelux b.v., Leiden, The Netherlands). Briefly, cells were seeded in flat bottom 96-well micro culture plates at a density of $3 \cdot 10^4$ cells/well in 100 μl medium. After overnight culture, medium was replaced with fresh medium containing the various experimental conditions. After 16 h, MTS assay was performed according to manufacturer's recommendations. Experimental apoptosis induction was quantified as the percentage apoptosis compared to medium control, which was set at 0% apoptosis. Each experimental and control group consisted of six independent wells.

Apoptosis induction assessed by loss of Mitochondrial Membrane Potential (ΔΨ)

Where indicated, apoptosis induction apparent from loss of ΔΨ was analyzed using the cell-permeant green-fluorescent lipophilic dye DiOC6 (Molecular Probes, Eugene, USA) as previously described[40]. In short, after 16 h treatment cells were harvested by centrifugation (300xg; 5 min) and incubated for 30 min at 37°C with fresh medium containing 0.1 μM DiOC6, washed twice with phosphate buffered saline (PBS), and analyzed using flow cytometry.

Immunoblot analysis of caspase activation and PARP cleavage

Where indicated, apoptosis induction apparent from caspase-8 and caspase-3 activation and PARP degradation was assessed by immunoblot analysis using antibodies against active caspase-8 (Cell signalling technology, Beverly, MA, USA), active caspase-3 (BD biosciences, San Jose, CA, USA), and PARP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) respectively. Briefly, cells were seeded in 6 well plates at a final concentration of $0.5 \cdot 10^6$ cells/ml and treated as indicated. Cells were harvested by centrifugation (2,000xg; 10 min), lysed in lysis buffer (20 mM Tris-HCl, 5.0 mM EDTA, 2.0 mM EGTA, 100 mM NaCl, 0.05% SDS, 0.50% NP-40, 1 mM PMSF, 10 μg/ml Aprotein, 10 μg/ml Leupeptin, pH 6.8), and sonicated on ice for 2x5 sec. Cleared supernatants were collected after centrifugation (15,000xg; 10min) and protein concentration was determined using Bradford method according to manufacturer's instructions (BioRad, Hercules, CA, USA). Samples were diluted 1:1 in standard SDS/PAGE-loading buffer containing 2-mercapto-ethanol and boiled for 10 minutes. Samples of 30 μg total protein were loaded and separated using 10% acryl amide SDS-PAGE, followed by electroblot transfer to nitrocellulose. Blots were incubated with the respective primary MAbs and appropriate HRPO-conjugated secondary antibodies. Specific binding of MAbs was detected using ECL (Roche diagnostics, Indianapolis, IN, USA). All antibody incubations were performed at room temperature for 1.5 h in PBS containing 5% bovine serum albumin, followed by 3 washes with PBS containing 0.1% Tween-20.

Distinctive fluorescent labelling of target and bystander cells

Differential cell membrane labelling of target and bystander cells was achieved using the Vybrant Multicolor Cell-Labelling kit (Molecular probes). EGP2-negative bystander cells were labelled with the red fluorescent dye DiI, while the corresponding EGP2-positive target cells were not labelled. Briefly, labelling was performed by incubation of bystander

cells ($1 \cdot 10^6$ cells/ml in serum free medium) with 5 μM DiI (37°C; 5 min) followed by three subsequent washes with medium (1,200xg; 5 min). Pellet was resuspended in medium whereupon the DiI-labelled bystander cells were mixed with non-labelled target cells at the target- to bystander ratios indicated at a final concentration of $0.5 \cdot 10^6$ cells/ml. Differential membrane fluorescent characteristics allow the target and bystander cells to be separately evaluated in mixed culture after treatment.

Distinctive quantification of apoptosis in target and bystander cells by ΔΨ

Non-labelled target cells and DiI-labelled bystander cells were mixed, at the indicated ratios, at a final concentration of $0.5 \cdot 10^6$ cells/well in a 12-well plate. After overnight culture, cell mixtures were treated with scFvC54:sTRAIL (300 ng/ml) for 16 h in the presence or absence of MAb MOC31 (5 μg/ml), caspase 8 inhibitor Z-IETD-FMK (1 μg/ml) (Calbiochem, San Diego, CA, USA), or TRAIL-neutralizing MAb 2E5 (1 μg/ml). The differential fluorescent characteristics of target and bystander cells were subsequently used to separately evaluate the amount of apoptosis induced in target and bystander cells by measuring ΔΨ with the fluorescent dye DiOC6 as described above.

FACS-sorting of target and bystander cells after treatment

FACS-sorting was applied to separate mixed target cells and bystander cells after treatment with scFvC54:sTRAIL. To this end, DiI-labelled bystander cells (Jurkat) were mixed with an equal amount of unlabelled target cells (Jurkat.EGP2) at a final concentration of $0.5 \cdot 10^6$ cells/ml. This mixed cell culture was treated with scFvC54:sTRAIL (300 ng/ml) for 6 h in the presence or absence of MAbs MOC31 (5 μg/ml) or 2E5 (1 μg/ml). After treatment, cell mixtures were collected and washed twice in fresh medium pre-cooled at 0°C. Subsequently, $2.5 \cdot 10^6$ cells of both the target and bystander cells were sorted using the MoFlo high-speed cell sorter. The sorted cells were found to be >99% pure and were separately analyzed for apoptotic features by immunoblot as described above.

Fluorescence microscopy of bystander apoptosis induction

Fluorescent microscopy was used to visualize bystander apoptosis induction in the adherent growing glioblastoma cell line U87MG. U87MG.EGP2 target cells, brightly expressing EGFP, were mixed at a 1:4 ratio with U87MG bystander cells at a final concentration of $0.5 \cdot 10^6$ cells/well on Lab-Tek chamber slides (Nalge Nunc int., Naperville, IL, USA). After overnight culture, spent medium was carefully aspirated and the mixed cell culture was subjected to treatment with scFvC54:sTRAIL (300 ng/ml) for 16 h, in the presence or

absence of MAb MOC31 (5 μg/ml) or MAb 2E5 (1 μg/ml), respectively. After treatment, apoptosis induction apparent from nuclear morphology was analyzed using the DNA binding dye Hoechst 33342 (Molecular probes). Both nuclear morphology and EGFP-fluorescence were visualized using a Quantimed 600S fluorescence microscope (Leica Camera Ag, Solms, Germany).

Quantification of innocent bystander apoptosis in isolated PBMCs

Leukocytes were isolated from EDTA anti-coagulated blood of healthy donors using the Ammonium Chloride method according to standard procedure. Briefly, blood was diluted 8-fold with cold Ammonium Chloride buffer and incubated for 10 min at 0°C, allowing the lysis of red blood cells. Subsequently, leukocytes were collected by centrifugation (300xg; 5 min). The above-described procedure was repeated to ensure complete lysis of all red blood cells. Isolated leukocytes were resuspended (RPMI, 10% human pool serum) and mixed at a target- to bystander ratio of 1:1 with target Jurkat.EGP2 cells that were labelled with the green fluorescent dye DiO (Molecular Probes). Mixed cultures were treated for 16 h with scFvC54:sTRAIL in the presence or absence of MAb MOC31 or MAb 2E5. The degree of apoptosis induction after treatment was analyzed by addition of the fluorescent DNA-binding dye Propidium Iodide (PI), and quantification of the percentage of PI-positive cells using flow cytometry.

Results

TRAIL-R expression in EGP2-transduced cell lines

Flow cytometric analysis of the retrovirally transduced cell lines Jurkat.EGP2, Ramos.EGP2, and U87MG.EGP2 revealed a strong homogeneous cell surface expression of EGP2 and intracellular EGFP fluorescence (data not shown). No significant differences in TRAIL receptor expression patterns were found between parental and EGP2-transduced cell lines (Table 1).

Target cell-restricted apoptosis induction by scFvC54:sTRAIL

EGP2-negative bystander cells (Jurkat, Ramos, U87MG) were not susceptible to apoptosis induction by scFvC54:sTRAIL. After prolonged treatment (16 h) with 300 ng/ml scFvC54:sTRAIL, cell cultures contained only low percentages of apoptotic cells (Fig.2A; 5%, 10%, and 3%, for Jurkat, Ramos, and U87MG, respectively). When treatment was performed in the presence of multimeric soluble EGP2 (3.5 μg/ml), which secondarily crosslinks scFvC54:sTRAIL, a strong increase in the percentages of apoptotic cells was

Table 1. Flow cytometric analysis of TRAIL receptor expression on target and bystander cells. EGP2-positive target cells and EGP2-negative bystander cells were analyzed for TRAIL receptor expression. Expression of TRAIL receptors was classified as not detectable (n.d.) when MFI was below 5 and subsequently in the following categories; 5<MFI<25 (*), 25<MFI<50 (†), 50<MFI<125 (‡).

Cell Line	TRAIL-R1	TRAIL-R2	TRAIL-R3	TRAIL-R4
Ramos	*	*	ND	†
Ramos.EGP2	*	*	ND	†
Jurkat	ND	*	ND	†
Jurkat.EGP2	ND	*	ND	†
U87MG	†	‡	†	†
U87MG.EGP2	†	†	†	†

observed (92%, 84%, and 70% for Jurkat, Ramos and U87MG, respectively).

Treatment of EGP2-positive target cells (Jurkat.EGP2, Ramos.EGP2, and U87MG.EGP2) with 300 ng/ml scFvC54:sTRAIL induced a strong increase in percentage of apoptotic cells (Fig.2B; 71%, 67%, 65%, respectively). Treatment of these EGP2-positive cells in the presence of EGP2-blocking MAb MOC31 strongly inhibited apoptosis induction (16%, 13%, and 5%, for Jurkat.EGP2, Ramos.EGP2 and U87MG.EGP2, respectively). Interestingly, although levels of TRAIL receptor expression were comparable for both parental and EGP2-transduced cell lines, the sensitivity to apoptosis induction by scFvC54:sTRAIL was somewhat reduced in the EGP2-transduced cell lines. Reduced sensitivity of EGP2-transduced cells may be related to the retroviral transduction procedure of these cell lines or to the ectopic overexpression of EGP2.

Distinctive quantification of apoptosis induction in target and bystander cells

When mixed cultures of Jurkat.EGP2 target cells and Jurkat bystander cells were treated with scFvC54:sTRAIL, strong apoptosis induction was observed in Jurkat.EGP2 target cells ranging from 55% at target to bystander ratio 7:3 to 20% at ratio 1:100 (Fig.3A). Apoptosis induction in Jurkat bystander cells ranged from 80% at ratio 7:3 to 17% at the remarkably low ratio 1:100 (Fig.3B). Treatment of bystander cells alone resulted in marginal induction of apoptosis (7%). Percentages of apoptotic cells were strongly reduced in both target and bystander cell populations when treatment was performed in the presence of EGP2-blocking MAb MOC31 (Fig.3A and B). Analogously, treatment with scFvC54:sTRAIL in the presence of TRAIL neutralizing MAb 2E5 completely abrogated apoptosis (Fig.3A and B). Furthermore, when mixed cultures of target and bystander cells were treated with the Mock-scFvH22:sTRAIL fusion protein, containing the antibody fragment domain scFvH22 of irrelevant specificity, no apoptosis induction was found in

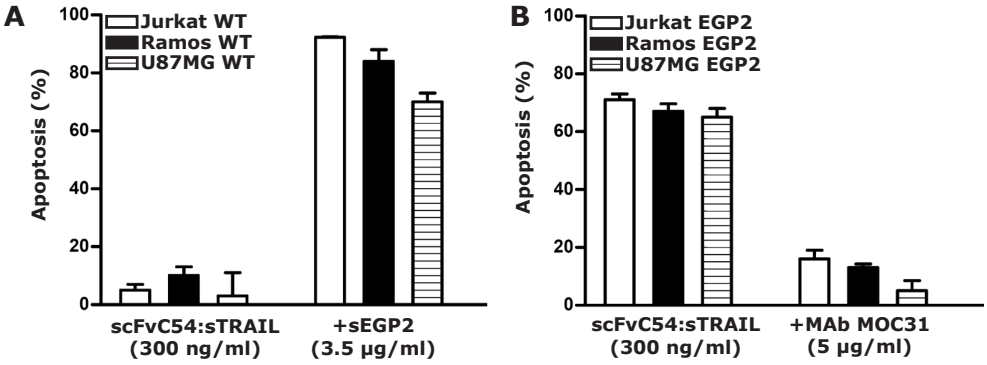

Fig.2. Target cell-restricted apoptosis induction by scFvC54:sTRAIL. A; EGP2-negative bystander cells (Jurkat, Ramos and U87MG) were treated with scFvC54:sTRAIL alone to determine sensitivity to apoptosis induction by non crosslinked scFvC54:sTRAIL. Additionally, cells were treated with scFvC54:sTRAIL in the presence of multimeric sEGP2, which secondarily crosslinks scFvC54:sTRAIL., to determine intrinsic sensitivity to crosslinked scFvC54:sTRAIL. **B;** EGP2-positive target cells (Jurkat.EGP2, Ramos.EGP2, and U87MG.EGP2) were treated with scFvC54:sTRAIL for 16 h in the presence or absence of target antigen-competing MAb MOC31. Apoptosis induction was assessed by MTS assay. All values indicated in the graphs are the mean + standard error of the mean of four independent experiments.

Jurkat.EGP2 target or Jurkat bystander cells (Fig.3C). Similar experiments with mixed cultures of Ramos.EGP2 and Ramos, and the adherent cell lines U87MG.EGP2 and U87MG, further confirmed that treatment with scFvC54:sTRAIL potently induced both target and bystander apoptosis, whereas treatment with Mock-scFvH22:sTRAIL did not lead to significant apoptosis induction in target or bystander cells (Fig.3C).

Target and bystander apoptosis induction is caspase-8 dependent

In a mixed culture of Ramos.EGP2 target cells and Ramos bystander cells (ratio 2:3), treatment with scFvC54:sTRAIL resulted in bystander apoptosis induction up to 65% (Fig.4A). Treatment in the presence of the specific caspase-8 inhibitor Z-IETD-FMK strongly inhibited apoptosis induction in both target and bystander cells (Fig.4A; 15% and 17%, respectively). Moreover, treatment of a mixed culture of Ramos.EGP2 target cells and bystander cells ectopically overexpressing the caspase-8 inhibitor c-FLIP$_L$ (Ramos.c-FLIP$_L$), revealed that Ramos.c-FLIP$_L$ bystander cells were largely resistant to the pro-apoptotic bystander effect of scFvC54:sTRAIL (Fig.4B; 17%). The residual induction of apoptosis in Ramos.c-FLIP$_L$ bystander cells was specifically inhibited by co-incubation of cells with MAb MOC31.

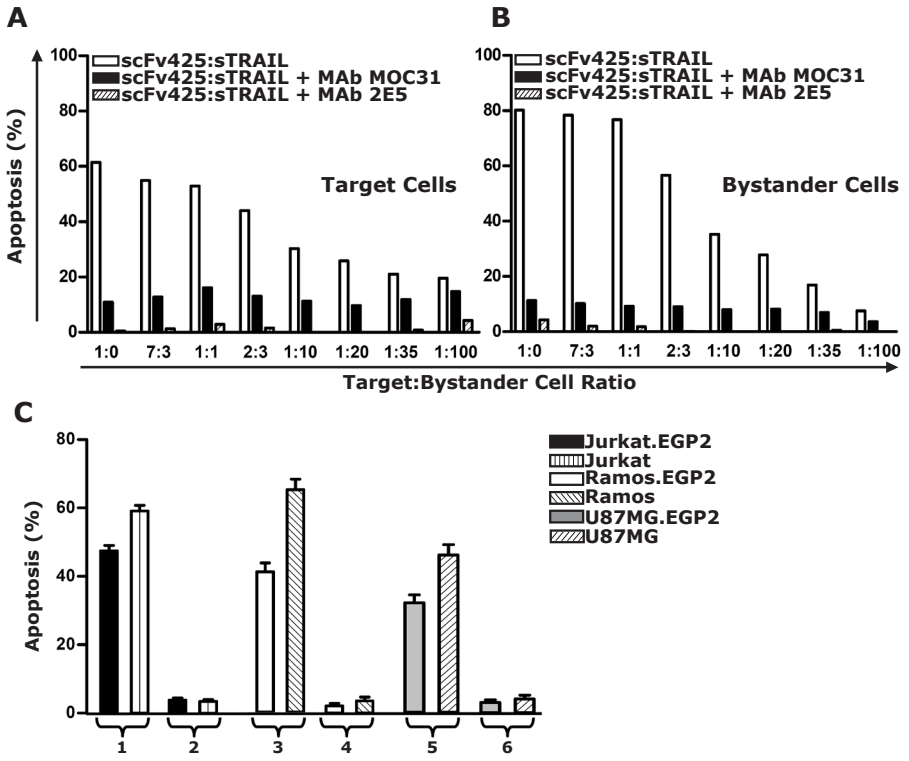

Fig.3. Separate evaluation of target cell and bystander cell apoptosis induction by scFvC54:sTRAIL. Jurkat.EGP2 target and Jurkat bystander cells were mixed at different target- to bystander ratios and treated for 16 h with 300 ng/ml scFvC54:sTRAIL in the presence or absence of MAb MOC31 (5 µg/ml) or MAb 2E5 (1 µg/ml). After treatment, cells were harvested and apoptosis induction was separately evaluated in **A;** Jurkat.EGP2 target cells and **B;** Jurkat bystander cells. **C;** Target antigen-dependent fratricide and bystander apoptosis induction. Mixed cultures of target and bystander cell combinations Jurkat.EGP2/Jurkat, Ramos.EGP2/Ramos and U87MG/U87MG.EGP2 (at target- to bystander ratio 2:3) were treated with 300 ng/ml scFvC54:sTRAIL (bars 1, 3 and 5), or with equal amounts of an scFv:sTRAIL fusion protein of irrelevant specificity (Mock-scFvH22:sTRAIL) (bars 2, 4 and 6). Apoptosis induction was separately evaluated in the target and bystander cells by loss of $\Delta\Psi$ as described in M&M section. All values indicated in the graphs are mean + standard error of the mean of four independent experiments.

Immunoblot analysis of FACS-sorted target and bystander cells

Post-treatment sorting of a mixed culture of Jurkat.EGP2 target and Jurkat bystander cells (ratio 1:1), allowed for the separate evaluation of apoptotic features in target and bystander cells. Treatment with scFvC54:sTRAIL induced a clear activation of caspase-8 and caspase-3 in both Jurkat.EGP2 target cells (Fig.5A, lane 2) and Jurkat bystander cells (Fig.5B, lane 2). Activation of caspase-3 was accompanied by cleavage of its target

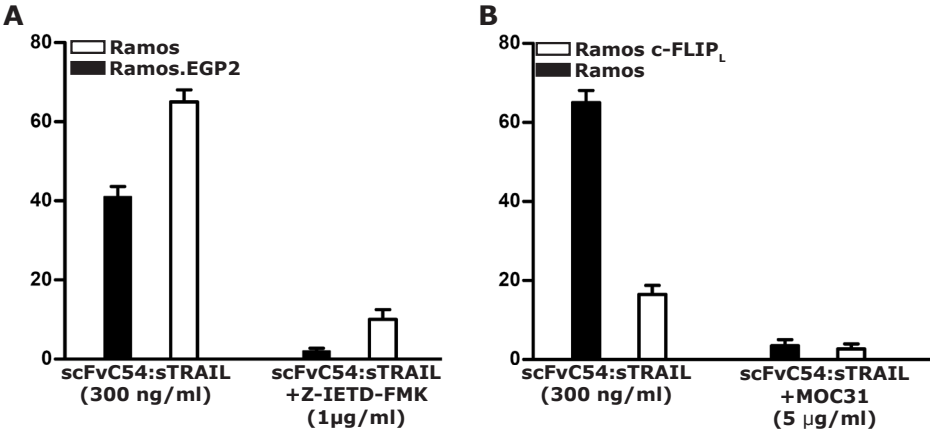

Fig.4. Target cell and bystander cell apoptosis induction by scFvC54:sTRAIL is caspase-8 specific. A; Ramos.EGP2 target cells and Ramos bystander cells were mixed at target- to bystander ratio 2:3 and treated with scFvC54:sTRAIL in the presence or absence of caspase-8 inhibitor Z-IETD-FMK. After 16 h, cells were harvested and apoptosis induction was separately evaluated by loss of ΔΨ in Ramos.EGP2 target cells and Ramos bystander cells. **B;** Ramos bystander cells ectopically over-expressing c-FLIP$_L$ are largely insensitive to bystander apoptosis induction. Ramos.EGP2 target cells were mixed at target- to bystander ratio 2:3 with either parental Ramos bystander cells or with Ramos bystander cells ectopically overexpressing c-FLIP$_L$ (Ramos.c-FLIP$_L$). Mixed cultures were subsequently treated with 300 ng/ml scFvC54:sTRAIL in the presence or absence of MAb MOC31, after which apoptosis induction was evaluated in bystander Ramos or Ramos.c-FLIP$_L$ by loss of ΔΨ. All values indicated in the graph are mean + standard error of the mean of four independent experiments.

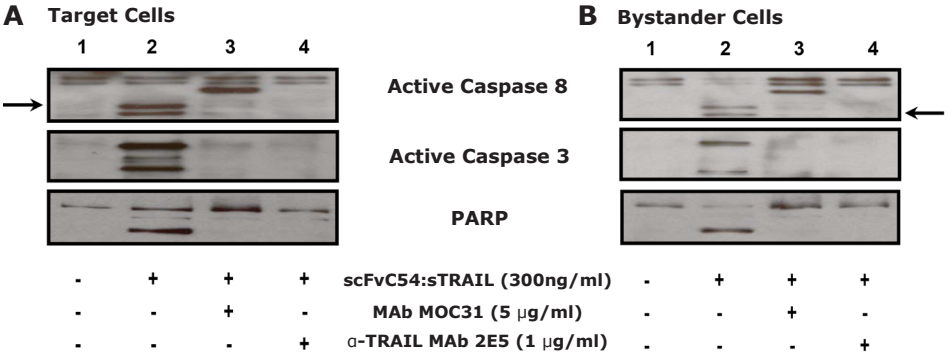

Fig.5. Separate evaluation of caspase activation and PARP cleavage in Jurkat.EGP2 target cells and Jurkat bystander cells. Jurkat.EGP2 target cells and Jurkat bystander cells were mixed at target to bystander ratio 1:1 and treated for 6 h with scFvC54:sTRAIL, in the presence or absence of MAb MOC31 or MAb 2E5. After treatment, Jurkat.EGP2 target and Jurkat bystander cells were separated by high-speed cell sorting, after which **A;** Jurkat.EGP2 target cells and **B;** Jurkat bystander cells were separately analyzed by immunoblot for caspase-8 activation, caspase-3 activation, and PARP degradation. Arrows indicate bands corresponding to cleaved caspase-8. Of note, in the caspase-8 blot of both Jurkat.EGP2 target cells and Jurkat bystander cells a specific band derived from the heavy chain of MAb MOC31 is also visible.

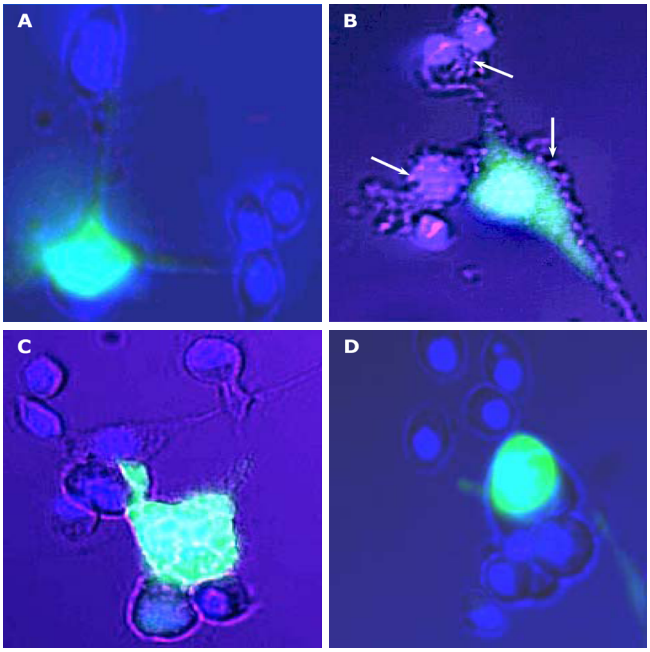

Fig.6. Visualization of bystander apoptosis by fluorescence microscopy. U87MG.EGP2 target cells and U87MG bystander cells were mixed at a target-to-bystander ratio of 1:4 and precultured on chamber slides. Subsequently, the mixed cultures were subjected for 16 hours to treatment with **(A)** medium, **(B)** scFvC54:sTRAIL (300 ng/ml), **(C)** MAb MOC31 (5 μg/ml) + scFvC54:sTRAIL, and **(D)** MAb 2E5 (1 μg/ml) + scFvC54:sTRAIL. After treatment, cells were stained using the nuclear stain Hoechst and analyzed for characteristic apoptotic morphology. Target and bystander cells could be distinctly discriminated due to the expression of green fluorescent protein by U87MG.EGP2 target cells only.

protein PARP (Fig.5A and B, lane 2). Treatment in the presence of MAb MOC31 or MAb 2E5 inhibited caspase activation and PARP cleavage in both target and bystander cells (Fig.5A and B, lane 3 and 4, respectively). Neither caspase activation nor PARP cleavage was observed when Jurkat bystander cells were treated in the absence of Jurkat.EGP2 target cells, even when treatment was prolonged to 24 h (data not shown).

Fluorescent microscopy of bystander apoptosis induction

Microscopic evaluation of untreated mixed cultures revealed that adherent U87MG.EGP2 target cells and U87MG bystander cells were interconnected by cellular protrusions (Fig.6A). Protrusions coming from U87MG.EGP2 target cells can be appreciated due to the EGFP-fluorescence present in the cytoplasm of these cells. When

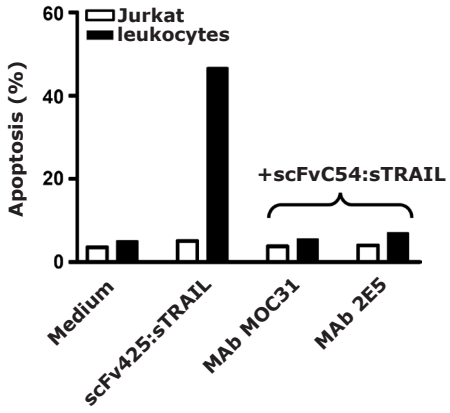

Fig.7. No innocent bystander cell apoptosis in isolated leukocytes. Isolated leukocytes were mixed with Jurkat.EGP2 target cells at a target-to-bystander ratio of 1:1. Mixed cultures were then treated for 16 hours with scFvC54:sTRAIL in the presence or absence of MAb MOC31 or MAb 2E5. Apoptosis induction was separately analyzed for leukocytes and Jurkat.EGP2 using PI staining, as described in Materials and Methods section.

a mixed culture of U87MG.EGP2 target cells and U87MG bystander cells (ratio 1:4) was treated with 300 ng/ml scFvC54:sTRAIL for 16 h, pronounced apoptotic morphological features such as membrane blebbing and nuclear condensation were visible in both target and bystander cells. The efficacy of the bystander effect was apparent from the fact that apoptotic morphology was observed in almost all U87MG bystander cells (Fig.6B). Identical treatment in the presence of either MAb MOC31 or MAb 2E5 strongly inhibited the appearance of apoptotic morphology in both U87MG.EGP2 target cells and U87MG bystander cells (Fig.6C and D).

No innocent bystander apoptosis in isolated PBMCs

Treatment of mixed cultures of isolated leukocytes (innocent bystander cells) and Jurkat.EGP2 cells with scFvC54:sTRAIL did not lead to any significant induction of apoptosis in the bystander leukocytes (Fig.7), whereas apoptosis of up to 46% was observed in Jurkat.EGP2 target cells. Apoptosis in Jurkat.EGP2 was specifically inhibited when treatment was performed in the presence of MAb MOC31 or MAb 2E5.

Discussion

It has been shown that crosslinking of TRAIL-receptors is crucial for the efficient induction of apoptosis in tumour cells. Previously, we reported on target cell-restricted fratricide apoptosis induction by the fusion protein scFvC54:sTRAIL, due to the efficient

crosslinking of agonistic TRAIL receptors TRAIL-R1 and TRAIL-R2[34]. In the present study we analyzed whether selective binding of scFvC54:sTRAIL to EGP2-positive tumour cells could further be used to crosslink TRAIL-receptors on neighbouring tumour cells devoid of EGP2 expression using mixed cell culture experiments. To this end, we selected a series of cell lines that represent three major human malignancies: acute lymphoblastic T cell leukemia (Jurkat), B cell lymphoma (Ramos), and Glioblastoma Multiforme (U87MG), all of which are EGP2-negative and generated EGP2-positive target cells thereof by retroviral transduction. Furthermore, we devised a method that allowed for distinctive evaluation of apoptosis in target and bystander cells.

All EGP2-negative bystander cell types used were fully resistant to prolonged treatment with scFvC54:sTRAIL (16h, 300 ng/ml). However, when mixed cultures of EGP2-positive target cells and corresponding EGP2-negative bystander cells were treated, potent pro-apoptotic effects of up to 80% apoptosis induction were achieved in EGP2-negative bystander cells (Fig.3A). Pro-apoptotic bystander activity of scFvC54:sTRAIL was observed for both suspension tumour cell types (Jurkat and Ramos) and for adherent U87MG glioblastoma cells (Fig.3C).

Treatment of mixed cultures containing as little as 1% of EGP2-positive target cells still showed significant apoptosis induction of up to 17% in EGP2-negative bystander cells. This clearly indicated that significant pro-apoptotic bystander activity of scFvC54:sTRAIL can be achieved at low target- to bystander cell ratios. Treatment in the presence of an EGP2-blocking antibody or a TRAIL-neutralizing antibody strongly inhibited apoptosis induction in both target and bystander cells at all ratios analyzed (Fig.3A and B). When treatment was performed using identical amounts of a scFv:sTRAIL fusion protein of irrelevant specificity (Mock-scFvH22:sTRAIL), no induction of apoptosis was observed (Fig.3C). Furthermore, apoptosis induction was specifically absent in bystander cells that ectopically over-expressed c-FLIP$_L$, a specific inhibitor of death receptor-induced apoptosis (Fig.4B). Immunoblot analysis of post treatment-sorted target and bystander cells demonstrated identical activation profiles of caspase-3 and caspase-8, and cleavage of PARP (Fig.5A and B). Together these results all indicated that both fratricide and bystander apoptosis induction by scFvC54:sTRAIL is mediated by target cell-dependent inter-cellular crosslinking of agonistic TRAIL-receptors.

Microscopic evaluation of a mixed culture (ratio 1:4) of adherent U87MG.EGP2 target cells and U87MG bystander cells treated with scFvC54:sTRAIL visualized pronounced apoptotic morphological features (nuclear condensation and membrane blebbing) in both target and bystander cells. The strong bystander effect observed here might partly be due to the

fact that U87MG cells have extensive cellular protrusions that appear to make multiple intracellular connections even to more distant cells (Fig.6A). Possibly, this particular cell morphology influences TRAIL-receptor crosslinking by scFvC54:sTRAIL between interconnected target- and bystander cells. It is tentative to speculate that scFv:sTRAIL treatment of target cells with more extensive cellular protrusions may induce apoptosis in more distant bystander cells.

As discussed above, we analyzed the pro-apoptotic bystander effect by scFvC54:sTRAIL down to extremely low target to bystander cell ratios. We noticed that when treatment was performed at ratios < 1:10, apoptosis induction in the target cells was partly diminished (Fig.3A). It appears that the presence of a vast majority of bystander cells reduces direct cellular contacts between EGP2-positive target cells, subsequently reducing fratricide apoptosis induction of these cells. The inhibitory effect of bystander cells on fratricide apoptosis induction in target cells was not observed at higher and possibly more realistic target-to-bystander cell ratios.

Previously, bystander effects have been observed in ADEPT (antibody-directed enzyme prodrug therapy)[41] and VDEPT (virus-directed enzyme prodrug therapy)[13,42], therapeutic approaches that target a non-human prodrug-converting enzyme into tumour cells and involve the transfer and diffusion of toxic metabolites from one cell to another. Usually, the toxic metabolites produced using these strategies cannot freely transit the cell membrane. Consequently, these bystander effects chiefly depend on GJIC between target and bystander tumour cells[14,43-45]. Unfortunately, most cancer cells lack functional GJIC. The bystander apoptosis activity described here for scFvC54:sTRAIL does not require internalization, enzymatic conversion, diffusion, or gap junctional intercellular communication (GJIC) between target and bystander cells.

An additional problem in both ADEPT and VDEPT appears to be the preferential killing of targeted cells due to their relative high intracellular concentration of the toxic metabolite, resulting in a decreased bystander effect. In contrast, the bystander activity of scFvC54:sTRAIL is likely to be maintained during the whole process of target cell apoptosis induction. Moreover, apoptosis of a given target cell can yield numerous minute apoptotic bodies with intact EGP2-positive cellular membranes. *In vitro*, target cell-derived apoptotic bodies displaying scFvC54:sTRAIL might continue to contribute to the crosslinking of TRAIL receptors and potentially disseminate the bystander effect to more distant tumour cells. The presence and subsequent contribution of such apoptotic bodies to the bystander effect studied here remains to be clarified. However, *in vivo* it is likely that phagocytosing cells of the immune system rapidly scavenge such apoptotic bodies

before additional bystander apoptosis induction is initiated.

We wondered whether the potent pro-apoptotic bystander effect of scFvC54:sTRAIL might also result in the killing of 'innocent bystander' cells such as normal blood cells. Therefore, we added freshly isolated leukocytes to various bystander experiments and found no significant signs of apoptosis induction in the various blood cell types (Fig.7). This indicates that at least in this experimental setting scFvC54:sTRAIL has retained its tumour selective apoptosis activity with no signs of innocent bystander apoptosis induction. Nevertheless, from the present study it cannot be excluded that scFvC54:sTRAIL might exert toxic or innocent bystander effects towards other normal cells and tissues. Toxicity studies of scFvC54:sTRAIL can possibly be performed in our human EGP2 transgenic mouse model[46] in which human EGP2 expression displays authentic expression patterns in mouse epithelia.

In conclusion, this is the first example of target cell-dependent bystander apoptotic activity by a scFv:sTRAIL fusion protein. Further development of this new principle is warranted for TRAIL and antibody-based therapy of human cancers that escape current antibody-based therapy due to heterogeneous target antigen expression.

Acknowledgements

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Simultaneous inhibition of EGFR signalling and enhanced activation of TRAIL-R-mediated apoptosis induction by an scFv:sTRAIL fusion protein with specificity for human EGFR.

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chapter 5

Abstract

EGFR-signalling inhibition by monoclonal antibodies (MAb) and EGFR-specific tyrosine kinase inhibitors has shown clinical efficacy in cancer by restoring susceptibility of tumour cells to therapeutic apoptosis induction. TRAIL is a promising anti-cancer agent with tumour-selective apoptotic activity. Here we present a novel approach that combines EGFR-signalling inhibition with target cell-restricted apoptosis induction using a TRAIL fusion protein with engineered specificity for EGFR. This fusion protein, scFv425:sTRAIL, comprises the EGFR-blocking antibody fragment scFv425 genetically fused to soluble TRAIL (sTRAIL). Treatment with scFv425:sTRAIL resulted in the specific accretion to the cell surface of EGFR-positive cells only. EGFR-specific binding rapidly induced the dephosphorylation of EGFR- and downstream mitogenic signalling, which was accompanied by c-FLIP$_L$ down-regulation and BAD dephosphorylation. EGFR-specific binding converted soluble scFv425:sTRAIL into a membrane-bound form of TRAIL that crosslinked agonistic TRAIL receptors in a paracrine manner, resulting in potent apoptosis induction in a series of EGFR-positive tumour cell lines. Co-treatment of EGFR-positive tumour cells with the EGFR tyrosine kinase inhibitor Iressa resulted in a potent synergistic pro-apoptotic effect, caused by the specific down-regulation of c-FLIP$_L$. Furthermore, in mixed culture experiments binding of scFv425:sTRAIL to EGFR-positive target cells conveyed a potent apoptotic effect towards EGFR-negative bystander tumour cells. The favourable characteristics of scFv425:sTRAIL, alone and in combination with Iressa, as well as its potent anti-tumour bystander activity indicate its potential value for treatment of EGFR-expressing cancers.

Introduction

The Epidermal Growth Factor Receptor (EGFR) is a transmembrane receptor tyrosine kinase comprising an extra-cellular ligand binding domain, a transmembrane lipophilic segment, and an intra-cellular tyrosine kinase domain[1,2]. Binding of its ligand EGF or TGFα results in EGFR-dimerization and activates the intrinsic protein tyrosine kinase. Activated EGFR concomitantly triggers signalling by the downstream mitogenic signal transduction pathways p44/42 MAPK and PI3K[3,4].

Normal EGFR signalling plays a pivotal role in organ development and repair. The important role of EGFR in the regulation of cell survival is underscored by the fact that aberrant EGFR activity strongly contributes to tumorigenesis in various tumour types. Aberrant activation of EGFR is associated with reduced recurrence-free or overall survival rates[5,6] and can arise from EGFR gene amplification, leading to high cell surface expression of over 10^6 EGFR molecules per cell, or alternatively, oncogenic mutation of EGFR. One

of the most frequent tumour specific mutant forms is the EGFRvIII, a mutant receptor commonly found in glioblastoma multiforma[7,8]. EGFRvIII possesses ligand-independent tyrosine kinase activity[9] and is associated with enhanced tumorigenicity in mice[10,11]. Very recently, mutations have been identified in the intra-cellular tyrosine kinase domain of EGFR in lung cancer patients that appear to activate anti-apoptotic pathways.

Several strategies have been developed to specifically inhibit aberrant EGFR signalling. Monoclonal antibodies, e.g. MAb C225 (Cetuximab™) and MAb 425[12,13], competitively inhibit the binding of natural ligands to the extracellular ligand-binding domain. Small molecule tyrosine kinase inhibitors, e.g Iressa (ZD1839 or Gefitinib™)[14,15], competitively inhibit with ATP for binding to the intracellular tyrosine kinase domain. The clinical efficacy of these agents appears to rely on multiple anti-cancer mechanisms, including inhibition of cell cycle progression, inhibition of metastasis, and an increase in the susceptibility of cells to apoptosis.

However, despite promising anti-tumour activity in clinical trials[16-19], both classes of EGFR-signalling antagonists do not appear to be curative. Therefore, additional EGFR-targeted strategies or combination with other therapeutic approaches are warranted. In this respect, strong synergistic tumoricidal effects have been reported for strategies in which EGFR-signalling antagonists are combined with radiation- or chemotherapy[17,19,20], and more recently, with the cytokine TRAIL[21].

TRAIL is normally present as a trimeric type II transmembrane protein (memTRAIL) on various immune effector cells. TRAIL specifically induces apoptosis in cancer cells[22] and virus-infected cells[23], without apparent apoptotic activity towards normal human cells. Homotrimeric memTRAIL initiates apoptosis by crosslinking of the agonistic receptors TRAIL-R1 and TRAIL–R2[24-27], leading to activation of the extrinsic apoptotic pathway via the Death Inducing Signalling Complex (DISC)[28-35]. Assembly of the DISC sequentially activates initiator caspases (caspase-8 or -10) and effector caspases (e.g. caspase-3, and -7) and ultimately ends in apoptotic cell death.

MemTRAIL can be proteolytically cleaved into a soluble form (sTRAIL). Several recombinant forms of sTRAIL have been generated that show strong tumoricidal activity *in vitro* and in xenografted mouse models without toxic side-effects[36-38]. Pharmacokinetic studies in cynamolgous monkeys and chimpanzees revealed no toxicity, thus further establishing the potential for clinical application of sTRAIL in cancer therapy.

However, TRAIL receptors are expressed in various tissues and, thereby, may potentially compete with tumour tissue for binding of i.v. applied sTRAIL. In addition, several papers described apoptotic activity of sTRAIL on various normal cells, including primary human hepatocytes[39], keratinocytes[40], prostate epithelial cells[41] and brain tissue[42]. Fortunately, the binding characteristics of sTRAIL for its receptors have typical "fast on/fast off" rates. Previously, we and others showed that sTRAIL can be genetically fused to a tumour

specific antibody fragment with "fast on/slow off" rates[43,44], resulting in the preferential binding to the pre-selected target antigen. In addition, the selected target antigen was selectively over-expressed compared to TRAIL-receptors on the tumour cell surface, thereby, further optimizing tumour cell-specific binding.

An additional advantage of antibody fragment targeted sTRAIL over conventional sTRAIL is its acquired capacity to activate TRAIL-R2. Conventional sTRAIL can efficiently activate TRAIL-R1 but not TRAIL-R2, the high affinity receptor that is activated only by membrane-bound TRAIL or sTRAIL secondarily crosslinked by antibodies[45,46]. Consequently, sTRAIL induces apoptosis less effectively in the many tumour types that predominantly express TRAIL-R2. Importantly, antibody fragment-mediated binding converts soluble scFv:sTRAIL into an artificial membrane bound form of TRAIL. Subsequently, a surplus of sTRAIL domains is available on the target cell surface for crosslinking of TRAIL-R2 on proximal tumour cells, resulting in enhanced target antigen-restricted reciprocal apoptosis.

Here, we report on a novel fusion protein, scFv425:sTRAIL, designed to combine EGFR-signalling inhibition with tumour-specific apoptosis induction by sTRAIL. ScFv425:sTRAIL consists of an antibody fragment derived from EGFR-blocking monoclonal antibody MAb 425[12] genetically fused to sTRAIL. Binding of scFv425:sTRAIL via the high affinity antibody fragment leads to specific accretion to the cell surface of EGFR-positive tumour cells. EGFR-specific binding of scFv425:sTRAIL was designed to rapidly inhibit EGFR signalling and, thereby, to sensitize cells to apoptosis[47]. EGFR-restricted binding of scFv425:sTRAIL restores full signalling capacity of scFv425:sTRAIL for both TRAIL-R1 and TRAIL-R2. Here we present a new therapeutic strategy combining EGFR-signal inhibition with simultaneous target antigen-restricted apoptosis induction by TRAIL. Taken together, the data described here warrant further clinical development of this novel fusion protein.

Materials and methods

Cell lines

The following cell lines were purchased from the ATTC (Manassas, USA); Jurkat (ALL T-cell line), A431 (epidermoid vulva carcinoma), A172, Hs683 (glioblastoma), SW948, and WiDr (colon carcinoma). Jurkat.EGFRvIII was generated by electroporation of Jurkat cells with plasmid pHβApr-1-neo/EGFRvIII (kind gift of Dr. D. Bigner, Duke University Medical Center, NC, USA), after which transfectants were selected by G418 selection (500 µg/ml, Gibco Life Technologies b.v. Breda, The Netherlands). Cell lines were cultured at 37°C, in a humidified 5% CO_2 atmosphere. Jurkat, SW948, and WiDr were cultured in RPMI 1640 (Cambrex Bio Science, Verviers, France) supplemented with 15% FCS. A431, HS683 and A172 were cultured in DMEM, 10% FCS, 4 mM L-glutamine (Cambrex Bio Science).

Monoclonal antibodies and inhibitors

TRAIL neutralizing MAb 2E5 was purchased from Alexis (10P's, Breda, The Netherlands). MAb 425 (kindly provided by Merck, Darmstadt, Germany) is a murine IgG2a with high binding affinity for the extra-cellular domain of EGFR and the mutant tumour specific variant EGFRvIII. MAb 425 blocks EGF binding to EGFR and competes with scFv425 for binding to the same epitope. Total caspase inhibitor Z-VAD-FMK, caspase-8 inhibitor Z-IETD-FMK, and caspase-9 inhibitor Z-LEHD-FMK were purchased from Calbiochem (San Diego, CA, USA). EGFR tyrosine kinase inhibitor Iressa was kindly provided by AstraZeneca Inc (Macclesfield, Cheshire, UK). PI3K inhibitor Wortmannin was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Final working concentrations of inhibitors were diluted in serum free medium from a stock of 10 mM in DMSO.

Production of scFv425:sTRAIL

Fusion protein scFv425:sTRAIL was constructed and produced essentially as described previously[43]. Briefly, In the first MCS of vector pEE14, the high-affinity antibody fragment scFv425 (Vh-(G4S)3-Vl format)[48] was directionally inserted using the unique *Sfi*I and *Not*I restriction enzyme sites. In the second MCS, a PCR-truncated 593 bp DNA fragment encoding the extracellular domain of human TRAIL (sTRAIL) was cloned in frame using restriction enzymes *Xho*I and *Hin*dIII, yielding plasmid pEE14-scFv425:sTRAIL. Expression plasmid pEE14-scFv425:sTRAIL was transfected into CHO-K1 cells using Fugene 6 reagent (Roche Diagnostics, Almere, The Netherlands) according to manufacturer's recommendations, after which transfectants were selected by the glutamine synthetase system as described[49]. Single cell sorting using the MoFlo high speed cell sorter (Cytomation, Fort Collins, USA) established clone 100F1, stably secreting 2,4 µg/ml scFv425:sTRAIL into the culture medium.

EGFR-specific binding of scFv425:sTRAIL

EGFR-specific binding of scFv425:sTRAIL was assessed by flow cytometry using the EGFR-positive tumour cell line A431 and the EGFR-negative cell line Jurkat. In short, $1 \cdot 10^6$ cells were incubated with scFv425:sTRAIL (300 ng/ml) in the presence or absence of MAb 425 (3 µg/ml). Specific binding of scFv425:sTRAIL was detected using PE-conjugated anti-TRAIL MAb B-S23 (Diaclone SAS, Besançon, France) and subsequent FACS analysis using an EPICS ELITE flow cytometre (Beckman Coulter, Mijdrecht, The Netherlands). Incubations were carried out for 45 min at 0°C and were followed by two washes with serum free medium.

Target cell-restricted induction of apoptosis by scFv425:sTRAIL

Target cell-restricted induction of apoptosis by scFv425:sTRAIL was assessed by analysis

of tumour cell viability, loss of mitochondrial membrane potential (Δψ), caspase-8 and -3 activation, and PARP cleavage/DFF degradation, as described in more detail below. Where indicated, treatment with scFv425:sTRAIL was performed in the presence or absence of MAb 425 (3 μg/ml) or MAb 2E5 (1 μg/ml).

Apoptosis assessed by viability assay

Cells were pre-cultured in a 96-well plate at a density of $3 \cdot 10^4$ cells/well. Subsequently, cells were treated for 16 h with the various experimental conditions in a final volume of 200 μl. Cell viability of adherent cell lines was determined by crystal violet staining (Sigma, Germany) as described previously[44]. Cell viability of suspension cell lines was determined using MTS assay (Promega Benelux b.v., Leiden, The Netherlands) according to manufacturer's recommendations. Experimental apoptosis induction was quantified as the percentage of apoptosis induction compared to base-line apoptosis in medium control, which was set at 0% apoptosis. Each experimental condition consisted of six independent wells.

Apoptosis assessed by loss of Mitochondrial Membrane Potential (Δψ)

Δψ was analyzed using the stain DiOC6 (Eugene, The Netherlands) as previously described[43]. Briefly, cells were pre-cultured in a 24-well plate at a concentration of $0.5 \cdot 10^6$ cells/well. Subsequently, cells were treated for 16 h with the various experimental conditions, after which cells were harvested and incubated for 20 minutes with DiOC6 (0,1 μM) at 37°C, harvested (300xg; 5 min.), resuspended in PBS, and analyzed by flow cytometry.

Immunoblot analysis

Cells were pre-cultured at $1.5 \cdot 10^6$ cells/well in a 6-well plate, after which cells were incubated with scFv425:sTRAIL in the presence or absence of MAb 425 or MAb 2E5 for the indicated time-points. Cell lysates were prepared as described previously[43]. Subsequently, 30 μg of lysate was separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose by electro blotting. Apoptosis signalling: Caspase activation was detected using antibodies directed against caspase-8 and active caspase-3 (Cell signalling, Beverly, MA, USA). PARP cleavage and DFF degradation was assessed using anti-PARP MAb (Santa Cruz Biotechnology Inc., Santa Cruz, Ca, USA) and anti-DFF MAb (Santa Cruz). Expression of c-$FLIP_L$ and BAD phosphorylation was determined using anti-c-$FLIP_L$ MAb clone NF6 (Alexis) and anti-phospho BAD antibody (Cell signalling). EGFR signalling: Expression levels of total and active EGFR were assessed using anti-total EGFR (Cell Signalling) and anti-activated EGFR (Tyr1173) (Santa Cruz). The different signal transduction pathways controlled by EGFR were analyzed with the phosphoERK1/2

sampler kit (Cell Signalling) and phospho-AKT sampler kit (Cell Signalling). Equal protein loading was assessed using anti-actin MAb (Boehringer Mannheim, Germany). Specific binding was visualized using appropriate secondary HRPO-conjugated antibody (DAKO Cytomation, Glostrup, Denmark) and chemoluminescence (Roche).

Differential quantification of apoptosis in target and bystander cells during mixed culture experiments

Differential cell membrane labelling of target and bystander cells was achieved using the Vybrant Multicolor Cell-Labelling kit (Molecular probes). Briefly, Jurkat.EGFRvIII target cells were labelled with the red fluorescent dye DiI. Labelling was performed by incubation of Jurkat.EGFRvIII cells ($1{\cdot}10^6$ cells/ml in serum free medium) with 5μM DiI (37°C, 5 min), followed by three washes with standard medium. DiI-labelled Jurkat.EGFRvIII target and non-labelled Jurkat bystander cells were mixed at the indicated ratios at a final concentration of $0.5{\cdot}10^6$ cells/well in a 24-well plate. After treatment, differential fluorescent characteristics of target cells and bystander cells were used to separately evaluate induction of apoptosis in both populations by Δψ as described above.

Synergistic induction of apoptosis by scFv425:sTRAIL and Iressa

Jurkat.EGFRvIII cells and A431 cells were simultaneously treated with suboptimal concentrations of scFv425:sTRAIL (80 ng/ml) and Iressa (250 and 2000 nM, respectively), unless indicated otherwise. Where indicated, cells were co-incubated with MAb 425 (3 μg/ml), MAb 2E5 (1 μg/ml), Z-VAD-FMK (1 μg/ml), Z-IETD-FMK (1 μg/ml), Z-LEHD-FMK (1 μg/ml), or PI3K inhibitor Wortmannin (10 μM). After 16 h treatment, apoptosis was assessed by Δψ as described above. Synergy was determined using the cooperativity index (CI), in which the sum of apoptosis induced by single-agent treatment is divided by apoptosis induced by combination-treatment. When CI<1, treatment was termed synergistic. The effect of single-agent and co-treatment of scFv425:sTRAIL and Iressa on apoptotic signalling and EGFR-signal transduction by PI3K and MAPK was assessed by immunoblot as described above.

Results

EGFR-specific binding of scFv425:sTRAIL

To assess whether scFv425:sTRAIL displayed specific and enhanced binding to EGFR-positive cells, A431 cells were incubated with scFv425:sTRAIL and analyzed for binding by flow cytometry. Strong binding of scFv425:sTRAIL was detected to the cell surface (Fig.1A, solid line), which could be specifically inhibited by pre-incubation with parental EGFR-blocking MAb 425 (Fig.1A, dashed line). In contrast, binding of scFv425:sTRAIL to

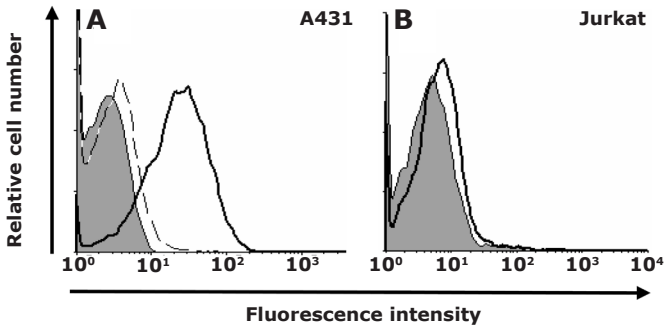

Fig.1. EGFR-specific binding of scFv425:sTRAIL. Binding of scFv425:sTRAIL was analyzed by flow cytometry using **A;** the EGFR-positive cell line A431 and **B;** the EGFR-negative cell line Jurkat. Cell lines were incubated with scFv425:sTRAIL alone (solid line) and, in the case of A431, were additionally pre-incubated with parental EGFR-blocking MAb 425 (dashed line). Binding of scFv425:sTRAIL was visualized using a monoclonal PE-conjugated anti-TRAIL antibody. Fluorescent intensity of unconditioned medium control is shown in solid fill.

TRAIL-receptors on the cell surface of EGFR-negative Jurkat cells was barely detectable (Fig.1B). The intensity of scFv425:sTRAIL binding directly correlated to the amount of cell surface expressed EGFR (data not shown).

EGFR-restricted induction of apoptosis by scFv425:sTRAIL

Treatment of EGFR-positive tumour cell lines with scFv425:sTRAIL (300 ng/ml) potently induced apoptosis (Fig.2A: A431; 66%, HS683; 85%, WiDr; 68%, SW948; 78%, A172; 70%, Jurkat.EGFRvIII; 82%), whereas EGFR-negative Jurkat cells were fully resistant to treatment (3%). Apoptosis was specifically inhibited when cells were co-incubated with MAb 425 or TRAIL-neutralizing MAb 2E5 during treatment with scFv425:sTRAIL (Fig.2B). Binding of scFv425:sTRAIL to EGFR and subsequent reciprocal activation of agonistic TRAIL-receptors in a paracrine fashion should also lead to apoptotic activity towards neighbouring EGFR-negative tumour cells. To investigate the presence of such a bystander effect, Jurkat.EGFRvIII target cells were mixed with Jurkat bystander cells (ratio 1:1) and treated with scFv425:sTRAIL. After treatment, bystander and target cells were separately assessed for apoptosis, which identified a potent bystander effect of 64% apoptosis in Jurkat bystander cells (Fig.2C). Apoptosis in Jurkat.EGFRvIII target cells reached approximately 50%. In both target cells and bystander cells, apoptosis was specifically inhibited by co-treatment with MAb 425 (4%) or MAb 2E5 (1%) (Fig.3C).

Inhibition of EGFR-signalling and subsequent sensitization to apoptosis by scFv425:sTRAIL treatment

Since scFv425:sTRAIL primarily binds via its EGFR-blocking antibody fragment scFv425,

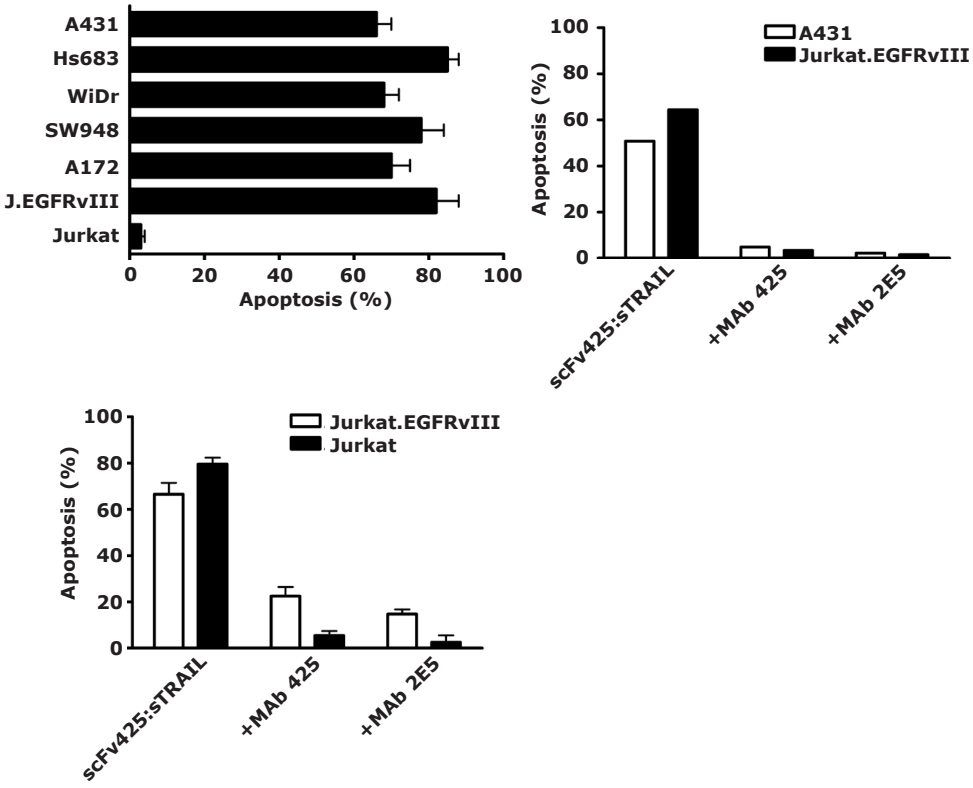

Fig.2. EGFR-restricted induction of apoptosis by scFv425:sTRAIL A; A panel of EGFR-positive tumor cell lines (A431, Hs683, WiDr, SW948, A172, and Jurkat.EGFRvIII) and an EGFR-negative cell line (Jurkat) was treated with 300 ng/ml scFv425:sTRAIL. Apoptosis induction was analyzed after 16 h using viability assay. **B:** A431 and Jurkat.EGFRvIII were treated with scFv425:sTRAIL (300 ng/ml) in the presence or absence of MAb 425 or TRAIL-neutralizing MAb 2E5. After 16 h, apoptosis induction was assessed by Δγ. **C:** Mixed cultures of Jurkat.EGFRvIII target cells and parental Jurkat bystander cells (ratio 1:1) were treated for 16 h with scFv425:sTRAIL in the presence or absence of MAb 425 or MAb 2E5. Differential fluorescent labeling of the target and bystander population was used to separately assess apoptosis by Δψ. Indicated values in bar graphs represent mean + standard error of the mean of three independent experiments.

the effect of scFv425:sTRAIL treatment on EGFR-signalling was determined. In A431 cells, scFv425:sTRAIL induced a rapid dephosphorylation of EGFR at Tyr 1173 within 10 min, while total EGFR levels remained constant (Fig.3A). The phosphorylation of EGFR observed during normal culture conditions is most likely due to a previously described TGFα-induced autophosphorylation loop[50]. Specific inactivation of EGFR signalling was accompanied by a small decrease in MAPK pathway activity, which was detected for MAPK

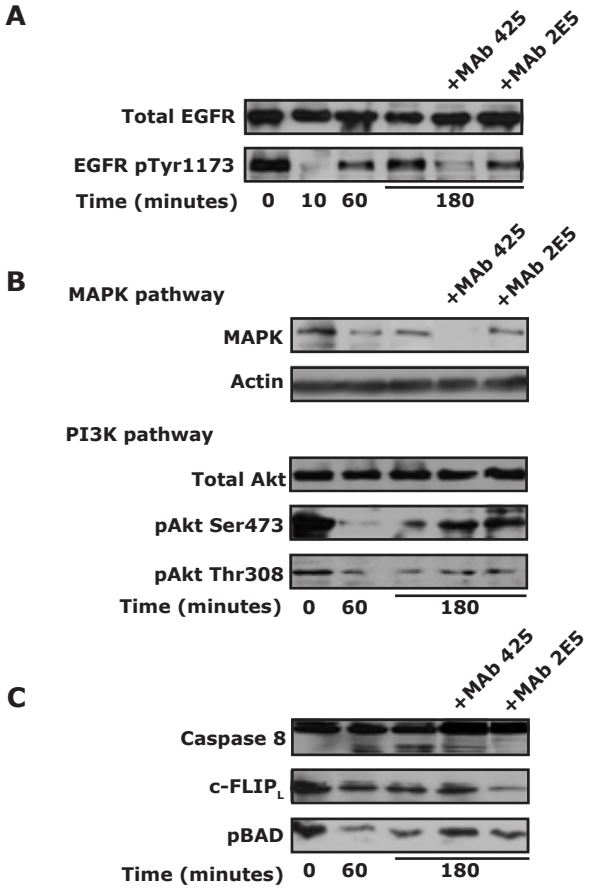

Fig.3. Inhibition of EGFR signaling and sensitization to apoptosis by scFv425:sTRAIL. A431 cells were challenged with 300 ng/ml scFv425:sTRAIL in the presence of 1 µg/ml cycloheximide for 10 min, 1h, and 3 h. At elapsed time-point 3h, cells were additionally treated with scFv425:sTRAIL in the presence or absence of MAb 425 or MAb 2E5. **A;** Cell lysates were analyzed for the amount of total and phosphorylated EGFR (pTyr1173). **B;** Cell lysates were analyzed for MAPK and PI3K pathway activity by measurement of phosphorylated p44/42 MAPK, total and phosphorylated Akt. Actin levels were determined to confirm equal protein loading. **C;** Cell lysates were analyzed for the apoptosis associated features of caspase-8 activation, the expression level of c-FLIP$_L$ and the phosphorylation status of BAD at residue Ser136.

at 1 and 3 h of treatment (Fig.2B). In addition, the PI3K pathway was markedly inhibited after 1 and 3 h of treatment, as measured by dephosphorylation of Akt at residues Tyr308 and Ser473 (Fig.2B), while total Akt levels remained constant.

Resistance to apoptosis by EGFR-signalling is mediated in part by its effect on the anti-apoptotic protein c-FLIP$_L$ and the phosphorylation of BAD via PI3K signalling. Since PI3K

signalling was inactivated by scFv425:sTRAIL treatment, c-FLIP$_L$ expression and BAD phosphorylation were investigated. At early time-points of 1 and 3 h of treatment, a decrease was detected in expression of the anti-apoptotic caspase 8 homologue c-FLIP$_L$ (Fig.3C), which coincided with the activation of caspase 8 (Fig.3C). Additionally, a marked decrease was observed in phosphorylation of BAD (Fig.3C), sensitizing the mitochondria to apoptosis.

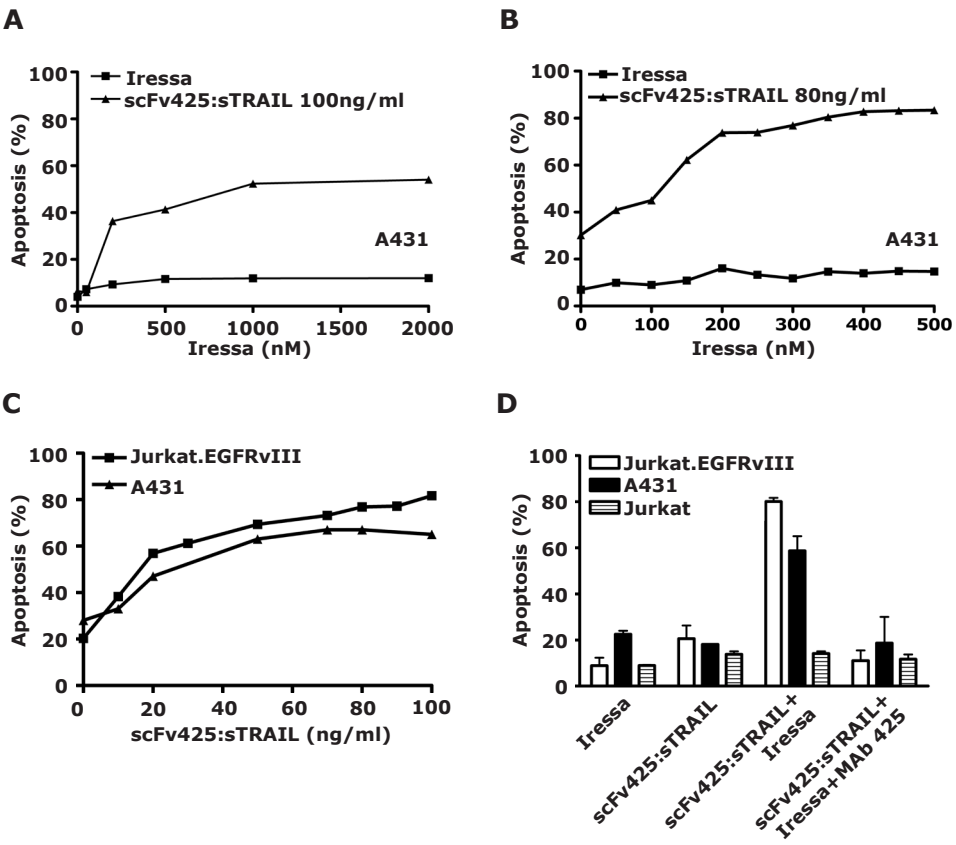

Fig 4. Synergistic target-cell restricted apoptosis induction by scFv425:sTRAIL and Iressa. **A;** A431 cells and **B;** Jurkat.EGFRvIII cells were treated with increasing concentrations of the EGFR-TKI Iressa in the presence or absence of a fixed concentration of scFv425:sTRAIL (80 ng/ml). **C;** Jurkat.EGFRvIII cells and A431 cells were treated with increasing concentrations of scFv425:sTRAIL in the presence of a fixed concentration of Iressa (250 nM and 2000 nM, respectively). **D;** Jurkat. EGFRvIII, A431, and Jurkat cells were treated alone, or with a combination of scFv425:sTRAIL and Iressa in the presence or absence of MAb 425. In all experiments, apoptosis was assessed by Δψ

Synergistic induction of apoptosis by scFv425:sTRAIL and Iressa

Previously EGFR-signalling inhibition was shown to synergistically enhance TRAIL-activity[21]. Therefore, potential synergistic effects of scFv425:sTRAIL with the EGFR-TKI Iressa was assessed on A431 cells and Jurkat cells positive for the mutant receptor EGFRvIII. Treatment of A431 cells with increasing concentrations of Iressa (250 - 2000 nM) and a fixed concentration of scFv425:sTRAIL (100 ng/ml) resulted in a dose-dependent synergistic increase in apoptosis (Fig.4A). Similar results, but with lower concentrations of Iressa (50 - 250 nM) and scFv425:sTRAIL (80 ng/ml), were obtained for Jurkat.EGFRvIII (Fig.4B). Dose-response curves of treatment with a fixed concentration of Iressa (250 and 2000nM, respectively) and increasing concentrations of scFv425:sTRAIL (up to 100 ng/ml) revealed a potent dose-dependent increase in apoptosis in both A431 and Jurkat.EGFRvIII cells already at 20 ng/ml of scFv425:sTRAIL (Fig.4C). The synergistic pro-apoptotic activity of scFv425:sTRAIL and Iressa was potently inhibited by co-treatment with MAb 425 (Fig.4C). Target antigen-negative Jurkat cells, subjected to the same experimental conditions, were fully resistant to treatment (Fig.4D). In control experiments with DMSO, alone or in combination with scFv425:sTRAIL, no significant induction of apoptosis was detected (data not shown).

Synergistic induction of apoptosis by scFv425:sTRAIL and Iressa is caspase 8-mediated

Next, the mechanism underlying the synergistic pro-apoptotic effect was investigated. Treatment of A431 cells and Jurkat.EGFRvIII cells with scFv425:sTRAIL and Iressa did not significantly alter TRAIL receptor expression (data not shown). Using specific caspase inhibitors, induction of apoptosis was found to be largely caspase-8 dependent, since the specific caspase-8 inhibitor Z-IETD-FMK inhibited apoptosis to levels observed for Iressa alone (Fig.5A). On the other hand, caspase-9 inhibition using Z-LEHD-FMK only had a minimal effect. Immunoblot analysis further revealed a strong activation of both caspase 8 and 3, resulting in PARP cleavage within 3 h of treatment with scFv425:sTRAIL and Iressa (Fig.5B). Single agent treatment only marginally activated caspase-8 and caspase-3 (Fig. 5B). Similar results were obtained when A431 cells were treated with scFv425:sTRAIL and Iressa (Fig.5C). The appearance of apoptotic features was specifically inhibited when treatment was performed in the presence of MAb 425 (Fig.5B and C).

Inhibition of EGFR signalling by co-treatment with scFv425:sTRAIL and Iressa

Simultaneous treatment of Jurkat.EGFRvIII with scFv425:sTRAIL and Iressa resulted in PI3K inactivation within 2 h in Jurkat.EGFRvIII, as measured by Akt dephosphorylation at Ser 473 (Fig.6A). No inhibition of MAPK signalling was observed in Jurkat.EGFRvIII, which is in line with a previous report showing that EGFRvIII specifically regulates PI3K activity[51]. At the concentrations used, single agent treatment had no effect on mitogenic

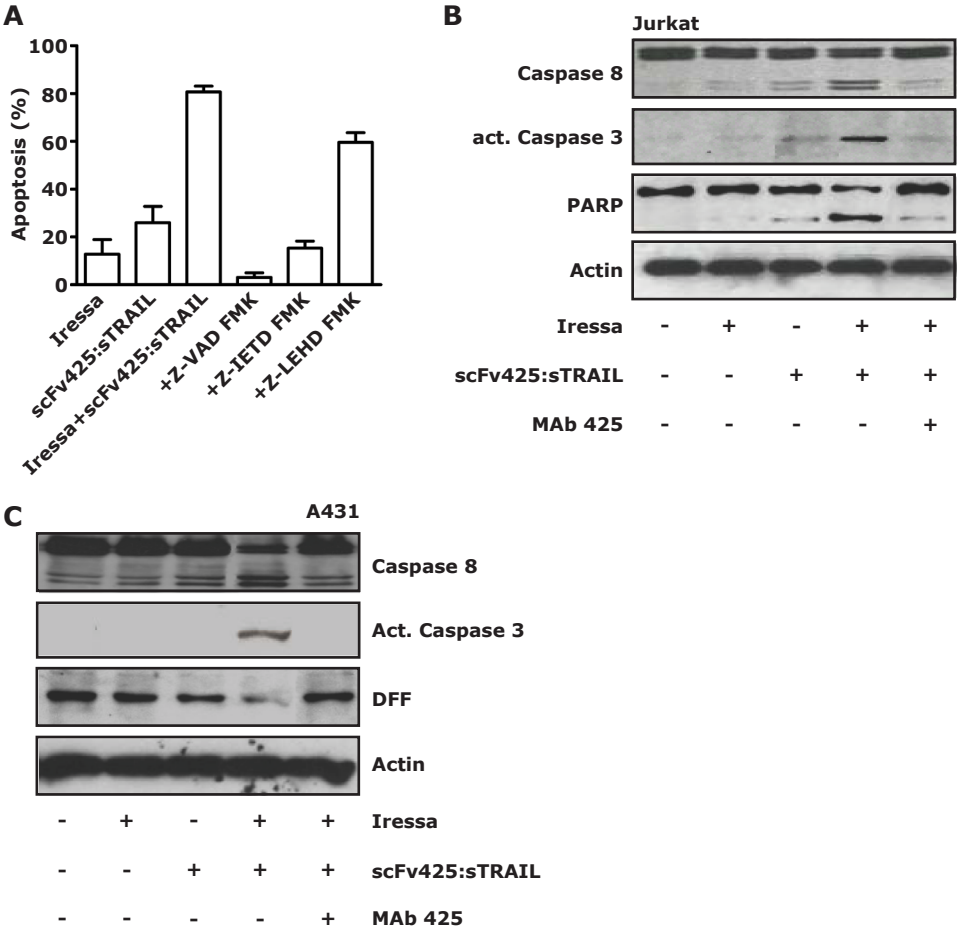

Fig.5. Synergistic induction of apoptosis by scFv425:sTRAIL and Iressa is caspase-8 mediated. A; Jurkat.EGFRvIII cells were subjected to single agent and combination treatment with scFv425:sTRAIL and Iressa. Co-treatment was performed in the presence of total caspase inhibitor Z-VAD-FMK, caspase-8 inhibitor Z-IETD-FMK, and caspase-9 inhibitor Z-LEHD-FMK. Apoptosis was determined by Δψ after 16 h of treatment. Values indicated are mean + standard error of the mean of three independent experiments. **B;** Jurkat.EGFRvIII cells and **C;** A431 cells were subjected to single agent- and co-treatment with scFv425:sTRAIL and Iressa for 3 h, after which cell lysates were analyzed for activation of caspase-8, activation of caspase-3, and PARP cleavage.

signalling in Jurkat.EGFRvIII (Fig.6A). The role of PI3K inhibition in the synergistic pro-apoptotic effect on Jurkat.EGFRvIII was confirmed by simultaneous treatment of Jurkat.EGFRvIII with the specific PI3K inhibitor Wortmannin and scFv425:sTRAIL, resulting in levels of apoptosis comparable to treatment with scFv425:sTRAIL and Iressa

(Fig.6B). For A431 cells, no effect of single agent and co-treatment was detected on PI3K signalling (Fig.6A). On the other hand, single agent treatment with Iressa already markedly inhibited MAPK signalling, while scFv425:sTRAIL treatment alone only had a

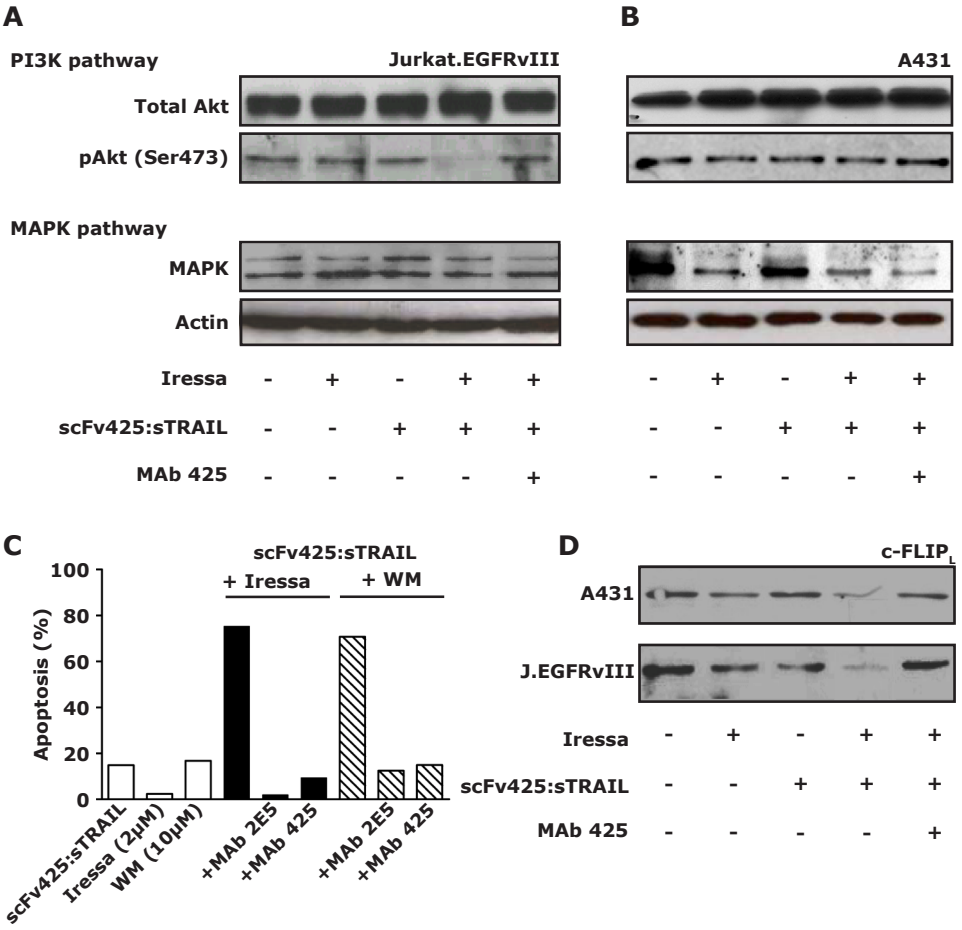

Fig.6. Inhibition of EGFR signaling by co-treatment with scFv425:sTRAIL and Iressa. Jurkat.EGFRvIII and A431 cells were treated either alone or with a combination of scFv425:sTRAIL and Iressa, in the presence or absence of MAb 425. After 2h treatment, PI3K pathway and MAPK pathway activity in **A;** Jurkat.EGFRvIII and **B;** A431 was assessed by immunoblot analysis of total Akt and active phosphorylated Akt, and phosphorylated MAPK, respectively. Equal protein loading was confirmed by actin staining. **C;** Jurkat.EGFRvIII was treated with scFv425:sTRAIL and either Iressa or the specific PI3K inhibitor Wortmannin, after which apoptosis induction was assessed by $\Delta\psi$. **D;** Cell lysates of A431 and Jurkat.EGFRvIII, treated alone or with a combination of scFv425:sTRAIL and Iressa, were analyzed for expression of the anti-apoptotic caspase 8 homologue c-FLIP$_L$.

minimal effect. Co-treatment of A431 also inhibited MAPK signalling, but to a similar extent as Iressa treatment alone (Fig.6B).

Treatment with scFv425:sTRAIL and Iressa induces c-FLIP$_L$ down regulation
Simultaneous treatment with scFv425:sTRAIL and Iressa markedly reduced the expression of c-FLIP$_L$ in both Jurkat.EGFRvIII and A431 cells (Fig.6D). To a lesser extent, treatment with Iressa alone down-regulated c-FLIP$_L$ in A431 cells, whereas in Jurkat.EGFRvIII no effect of single agent treatment was seen. Treatment in the presence of MAb 425 prevented down regulation of c-FLIP$_L$ in both cell lines.

Discussion

EGFR-signalling inhibition by EGFR-blocking monoclonal antibodies and small molecule tyrosine kinase inhibitors is a promising therapeutic approach that can restore the susceptibility of tumour cells to apoptosis induction. Here we describe a novel therapeutic approach in which EGFR-signalling inhibition is combined with target cell-restricted apoptosis induction using the new fusion protein scFv425:sTRAIL. Fusion protein scFv425:sTRAIL, comprising EGFR-blocking antibody fragment scFv425 genetically fused to sTRAIL, clearly accreted at the cell surface of EGFR-positive cells, which was specifically abrogated by pre-incubation with parental EGFR-blocking MAb 425. Together with the barely detectable binding of scFv425:sTRAIL to cognate TRAIL receptors on EGFR-negative cells, these data provide strong evidence for the enhanced binding specificity of scFv425:sTRAIL for EGFR-positive tumour cells.
Interestingly, high concentrations of parental MAb 425 were required to competitively inhibit binding of scFv425:sTRAIL, implying a high affinity of scFv425:sTRAIL for EGFR. Previously, we demonstrated that eukaryotically expressed scFv:sTRAIL is produced as a soluble homogeneous trimer[43]. Although not investigated here, such a stable trimeric form would provide a logic explanation for the strong binding observed on A431 cells. Trimeric scFv425:sTRAIL contains three identical antibody fragment domains and will, therefore, benefit from an associated enhanced avidity effect. Enhanced avidity has been shown to improve the *in vivo* tumour-targeting efficacy in several antibody-based strategies[52,53]. The above-described enhanced binding specificity and avidity of scFv425:sTRAIL may help increase tumour cell retention and reduce the total dose required to obtain a therapeutic effect.
Treatment with scFv425:sTRAIL potently induced apoptosis in EGFR-positive tumour cells that was specifically abrogated by co-incubation with parental MAb 425. When combined with the absence of apoptotic activity on EGFR-negative Jurkat cells, this established EGFR-specific binding of scFv425:sTRAIL as a critical component of its apoptotic activity. Interestingly, the appearance of apoptotic features, such as processing of caspase 8, was

preceded by the specific dephosphorylation of EGFR, and coincided with dephosphorylation of the PI3K signal transduction pathway and to a lesser extent the MAPK signal transduction pathway.

This rapid inactivation of EGFR-signalling clearly points to a role for EGFR inhibition in scFv425:sTRAIL-induced apoptosis. One of the main regulators of TRAIL sensitivity, the anti-apoptotic caspase-8 homologue c-FLIP$_L$[54-56], has previously been shown to be regulated by PI3K signalling[57,58]. In A431 cells, inactivation of PI3K signalling was accompanied by a decrease in expression of c-FLIP$_L$ after 1 and 3 h of treatment. Besides regulating c-FLIP$_L$ expression, PI3K signalling also influences the phosphorylation status of BAD[59,60]. In A431 cells, a marked dephosphorylation of BAD was detected after 1 and 3 h. Therefore, inhibition of PI3K signalling appears to facilitate caspase 8 activation, by down-regulating c-FLIP$_L$, and sensitizes the mitochondria to induction of apoptosis, by dephosphorylation of BAD.

In addition to PI3K inhibition, dephosphorylation of the MAPK signal transduction pathway was detected after 1 and 3 h of treatment with scFv425:sTRAIL. Previously, MAPK activation was shown to protect against TRAIL-induced apoptosis by a mechanism occurring at or above the level of caspase-8 processing, which did not involve c-FLIP$_L$[47]. Conversely, although not formally proven here, MAPK inhibition could sensitize tumour cells towards scFv425:sTRAIL-induced apoptosis at or above the level of caspase 8 processing.

From the above-discussed data, a model for the apoptotic activity of scFv425:sTRAIL can be formulated (for schematic representation see Fig.7). First, binding of scFv425:sTRAIL leads to accretion at the cell surface of EGFR-positive tumour cells only. Subsequently, EGFR-specific binding inhibits EGFR mitogenic signalling via PI3K and MAPK and, thereby, sensitizes tumour cells to apoptosis by e.g. down-regulation of c-FLIP$_L$ and BAD dephosphorylation. Concomitantly, membrane-bound scFv425:sTRAIL induces apoptosis by reciprocal crosslinking of agonistic TRAIL-receptors on neighbouring EGFR-positive tumour cells.

Paracrine activation of TRAIL-receptors by scFv425:sTRAIL is not necessarily restricted to EGFR-positive tumour cells but can also be directed towards neighbouring tumour cells devoid of target antigen. In a recent report, we described a potent anti-tumour bystander effect for an scFv:sTRAIL fusion protein with specificity for the carcinoma-associated cell surface target antigen EGP2[61]. Here, we show that scFv425:sTRAIL also potently induced apoptosis in EGFR-negative bystander Jurkat cells during mixed culture experiments with Jurkat.EGFRvIII cells. This pro-apoptotic bystander effect might help reduce the appearance of therapy-resistant recurrences emerging after seemingly successful treatment, as has been reported for conventional MAb-based therapy[62,63].

In a recent report, the synergistic effect of combined EGFR-targeting with the anti-EGFR monoclonal antibody cetuximab and the EGFR-specific tyrosine kinase inhibitor

Iressa was described[64]. Together with the fact that TRAIL has been shown to synergize with anti-EGFR agents[20], this provided a strong rationale for the combination of scFv425:sTRAIL treatment with Iressa. Potent synergistic induction of apoptosis was observed in both wild-type EGFR-positive A431 cells and EGFRvIII-positive Jurkat.EGFRvIII cells upon treatment with scFv425:sTRAIL and the Iressa. The synergistic pro-apoptotic effect of scFv425:sTRAIL and Iressa was fully EGFR-restricted and TRAIL-mediated and did not involve modulation of TRAIL receptor expression. Interestingly, inhibition of caspase 8 activity, by a specific caspase 8 inhibitor, reduced apoptosis induction by scFv425:sTRAIL and Iressa to the levels observed during Iressa treatment alone. On the other hand, caspase 9 inhibition only had a minimal effect on apoptosis induction. These data point to an increased processing of caspase 8 as the main cause for the synergistic pro-apoptotic effect with no or only minimal involvement of the mitochondrial route of apoptosis.

When cells were subsequently analyzed for expression of c-FLIP$_L$, the expression level of which is an important regulator of caspase 8 processing, a marked down regulation in both Jurkat.EGFRvIII and A431 was observed within 3 h of combination treatment with scFv425:sTRAIL and Iressa. Down-regulation of c-FLIP$_L$ coincided with the time of caspase-8 activation and was preceded by inactivation of the PI3K pathway in Jurkat. EGFRvIII cells. In A431 cells, combination treatment significantly inhibited MAPK-signalling but only to a similar degree as that observed during Iressa treatment alone.

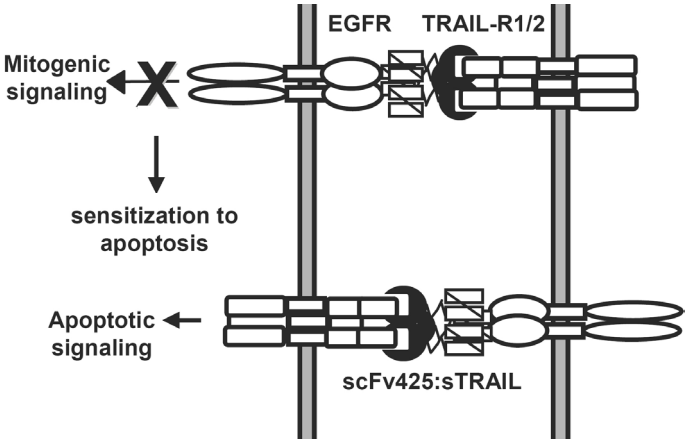

Fig 7. Schematic model of the apoptotic activity of scFv425:sTRAIL. Antibody fragment binding of scFv425:sTRAIL to EGFR inhibits mitogenic signaling by this receptor and its downstream signaling pathways and, thereby, sensitizes tumor cells to apoptosis. Furthermore, antibody fragment binding to EGFR immobilizes soluble scFv425:sTRAIL on the cell surface of EGFR-positive tumor cells and converts soluble scFv425:sTRAIL into a membrane bound form that can efficiently initiate apoptosis by crosslinking of the agonistic TRAIL receptors TRAIL-R1 and TRAIL-R2.

Based on these results, it can be concluded that the synergistic pro-apoptotic effect largely depends on the specific down-regulation of c-FLIP$_L$. For EGFRvIII positive Jurkat cells, down-regulation of c-FLIP$_L$ is a consequence of PI3K inhibition. In A431 cells, MAPK dephosphorylation may play a role but the exact mechanism remains to be elucidated.
In conclusion, we report for the first time on a recombinant fusion protein that combines the tumoricidal effect of EGFR-signal inhibition with target cell-restricted apoptosis induction. The unique characteristics of scFv425:sTRAIL described here indicate its potential therapeutic value, alone and in combination with EGFR tyrosine kinase inhibitor Iressa, for the treatment of EGFR and EGFRvIII expressing human cancers.

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chapter 6

Target cell-restricted apoptosis induction of acute leukemic T-cells by a recombinant TRAIL fusion protein with specificity for human CD7.

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Abstract

Current treatment of human T-cell Leukemia and lymphoma is predominantly limited to conventional cytotoxic therapy and is associated with limited therapeutic response and significant morbidity. Therefore, more potent and leukemia-specific therapies with favourable toxicity profiles are urgently needed. Here, we report on the construction of a novel therapeutic fusion protein, scFvCD7:sTRAIL, designed to induce target antigen-restricted apoptosis in human T-cell tumours. ScFvCD7:sTRAIL consists of the death inducing ligand TRAIL genetically linked to an scFv antibody fragment specific for the T-cell surface antigen CD7. Treatment with scFvCD7:sTRAIL induced potent CD7-restricted apoptosis in a series of malignant T-cell lines, while normal resting leukocytes, activated T-cells, and vascular endothelial cells (HUVECs) showed no detectable apoptosis. The apoptosis-inducing activity of scFvCD7:sTRAIL was stronger than that of the immunotoxin scFvCD7:ETA. In mixed-culture experiments with CD7-positive and CD7-negative tumour cells, scFvCD7:sTRAIL induced very potent bystander apoptosis of CD7-negative tumour cells. *In vitro* treatment of blood cells freshly derived from T-acute lymphoblastic leukemia (ALL) patients resulted in marked apoptosis of the malignant T-cells that was strongly augmented by vincristin. In conclusion, scFvCD7:sTRAIL is a novel recombinant protein causing restricted apoptosis in human leukemic T-cells with low toxicity for normal human blood and endothelial cells.

Introduction

In the last few decades, the treatment outcome of patients with leukemia and lymphoma has significantly improved. Nonetheless, only a minority of patients with T-cell ALL or peripheral T-cell lymphoma (PTCL) achieve long-term tumour-free survival[1]. Conventional cytotoxic therapy in these diseases is usually associated with substantial side-effects and limited response. Therefore, more potent targeted therapies with greater specificity and favourable toxicity profiles are needed in order to increase the so-far unsatisfactory treatment success of human T-cell tumours.

Recently, several leukemia-targeted therapeutic agents have been developed, including naked monoclonal antibodies (MAb), MAb-toxin conjugates, radioimmunoconjugates, and small molecules inhibiting key cellular functions such as tyrosine kinases. The research on many of these agents is still in early phases. Clinical experience with therapeutic antibodies in T-ALL is limited to the anti-CD3 MAb OKT3[2], which produced only a transient anti-tumour effect, while in more mature T-cell lymphoma, antibodies to CD52 (CAMPATH-1H)[3] and to CD25[4] have been used with considerable efficacy.

Currently, several CD7 MAb-toxin conjugates are evaluated in pre-clinical studies and

clinical trials, some with promising results[5-8]. However, targeted strategies using MAb-toxin conjugates can be severely hampered by toxin-related side effects, such as severe hepatic veno-occlusive disease frequently observed during treatment of AML with the anti-CD33 MAb-calicheamicin conjugate gemtuzumab-ozogamicin (GO, Mylotarg™)[9,10]. Thus, the development of antibody-derived therapeutic agents with strongly improved toxicity profiles is urgently needed.

A promising candidate for safe and cancer-restricted induction of apoptosis is the death-inducing ligand TRAIL. TRAIL is a recently identified member of the TNF family of death inducing ligands and shows selective apoptotic activity towards a variety of tumour cell types without toxicity for normal cells[11,12]. Native TRAIL is expressed as a homotrimeric type II transmembrane protein (memTRAIL) that can be proteolytically cleaved into soluble homotrimeric TRAIL (sTRAIL). Various forms of sTRAIL have been generated by recombinant DNA technology all showing potent *in vitro* and *in vivo* anti-tumour effects[13-15]. TRAIL binds to an elaborate receptor system comprising at least two agonistic receptors, TRAIL-R1 and TRAIL-R2[16-18], and two antagonistic receptors, TRAIL-R3 and TRAIL-R4[19-21]. The various TRAIL receptors are widely expressed on a variety of normal tissues and malignant cell types. Initially, TRAIL-R3 and –R4 were thought to act as decoy receptors, protecting normal and TRAIL-resistant tumour cells from apoptosis. However, recent reports show no correlation between TRAIL-sensitivity and expression of either TRAIL-R3 or TRAIL-R4[22,23]. Consequently, the mechanism for the tumour-selective activity of TRAIL remains elusive.

Clustering of TRAIL-R1 and -R2 by TRAIL leads to formation of the death-inducing signalling complex (DISC)[24-26]. The DISC includes the adaptor protein FADD and the initiator pro-caspases-8 or -10[24,27-29]. Efficient DISC assembly results in concomitant activation of initiator and effector caspases (e.g. caspases-3, -6, -7) and ultimately leads to apoptotic cell death.

Recently, it was shown that TRAIL receptors 1 and 2 have quite distinct crosslinking requirements for the initiation of apoptosis[30]. TRAIL-R2 signals apoptosis only after efficient receptor crosslinking by either native memTRAIL, aggregated sTRAIL variants, or by sTRAIL preparations secondarily crosslinked by antibodies. Apoptosis signalling by TRAIL-R1 was largely independent of the receptor crosslinking characteristics of a particular form of sTRAIL[30]. Furthermore, TRAIL-R2 had superior binding affinity for TRAIL[31], resulting in predominant binding of sTRAIL to TRAIL-R2 over TRAIL-R1.

Differential expression of TRAIL-R1 and -R2 has been described for various tumour types, usually with TRAIL-R2 being the most prevalent. Consequently, tumour cells predominantly expressing TRAIL-R2 are relatively insensitive to treatment with homogeneous trimeric sTRAIL preparations.

The tumour-selective activity of the various sTRAIL preparations was shown to critically

rely on their respective state of aggregation. High molecular weight sTRAIL aggregates in solution generated significant apoptotic activity to certain normal cell types[32]. Preparations containing only homogeneous non-aggregated homotrimeric forms of sTRAIL showed more authentic tumour-selective pro-apoptotic activity[33].

Therefore, to fully exploit the therapeutic potential of TRAIL, several characteristics of both the TRAIL receptor system and sTRAIL should be taken into account. First, the wide spread expression of the various TRAIL receptors throughout the human body. Second, the differential binding affinities and crosslinking requirements of the agonistic receptors TRAIL-R1 and TRAIL-R2, and third, the solution behavior of the sTRAIL preparation. Previously, we and others[34,35] demonstrated that these criteria can be largely met by genetically fusing sTRAIL to a tumour-specific recombinant antibody fragment. This fused sTRAIL, scFv:sTRAIL, was selectively directed to a pre-determined target antigen and was deposited on the cell surface of target cells only. Target antigen bound scFv:sTRAIL acquired TRAIL-receptor activating properties resembling that of native memTRAIL. Local accumulation and activation of this TRAIL construct and the corresponding receptors greatly improved its therapeutic potential.

Here we describe a novel fusion protein, designated scFvCD7:sTRAIL, that contains a scFv antibody fragment specific for human CD7, a cell surface glycoprotein abundantly expressed on most T cell malignancies and approximately 10% of acute myeloid leukemias (AML)[36-39]. Expression of CD7 on normal blood cells is limited to T- and myeloid cells in early hematopoietic cell ontogeny, thymocytes, NK cells, and a large distinct subset of peripheral blood T-cells[40-44]. Fusion protein scFvCD7:sTRAIL shows enhanced and target antigen-restricted apoptotic activity towards human T-ALL cells with no toxicity to normal human blood and endothelial cells.

Material and methods

Monoclonal antibodies, scFv antibody fragment, and scFvCD7:ETA

MAb TH69 is a murine IgG1 with specificity for human CD7[45]. DNA encoding the anti-CD7 scFv 3A1F[46] was kindly provided by Dr. Chris Pennell, Department of Laboratory Medicine and Pathology, University of Minnesota. MAb TH69 and scFvCD7 compete for binding to the same epitope on the extracellular domain of human CD7. The immunotoxin scFvCD7:ETA comprises an anti-CD7 scFv genetically linked to pseudomonas Exotoxin-A (ETA)[5]. TRAIL-neutralizing MAb 2E5 was purchased from Alexis (Kordia Life Sciences, Leiden, The Netherlands).

Chemotherapeutics

Vincristin (USPC Inc, Rockville, MD, USA), 1 mg/ml in PBS. UCN01 (provided by Kyowa Hakko Europe GmbH, Düsseldorf, Germany), 10 mM in DMSO. Cycloheximide (CHX),

(Sigma), 100 mg/ml in DMSO. Actinomycin D (Sigma), 2 mg/ml in ethanol. Final working concentrations were prepared by serial dilutions of stocks solutions in serum free medium.

Cell lines

Human T-ALL cell lines Jurkat, CEM, MOLT-16 (all CD7-positive) and the human B-cell lymphoma Ramos (CD7-negative), were purchased from the ATCC (Manassas, USA). CD7-positive Ramos cells were generated by transfection of Ramos cells with plasmid pSecTag/HygroC-CD7 and selection of CD7-positive transfectants using Hygromycin B (500 µg/ml) followed by flowcytometric cell sorting. Transfection of Ramos with CD7 did not alter TRAIL-receptor or c-FLIP expression. All cell lines were cultured in RPMI (Cambrex, New Jersey, New Hampshire, USA) supplemented with 15% FCS, at 37ºC in humidified 5% CO_2 containing atmosphere.

Isolation of leukocytes, PBLs, activated T-cells, and HUVECs

Leukocytes were isolated from whole blood of healthy donors using the Ammonium Chloride method[47]. Peripheral blood lymphocytes (PBLs) were isolated from whole blood of healthy donors by standard density gradient centrifugation procedures (Lymphoprep, Axis-Shield PoC As., Oslo, Norway). Freshly isolated resting PBLs were resuspended at $2.0x10^6$ cell/ml in RPMI, supplemented with 10% human pooled serum. Activated T-cells were generated by incubation of freshly isolated PBLs with anti-CD3 MAb (0.5 µg/ml) for 72 h, followed by IL2 stimulation (100 ng/ml) for 48 h. Human umbilical vein endothelial cells (HUVEC) were isolated as previously described[48]. HUVEC cells were used before culture passage number four and, for experiments, were pre-cultured in 6 well plates at 60% confluency.

Construction of scFvCD7:sTRAIL

Eukaryotic expression plasmid pEE14scFv:sTRAIL was generated for the rapid construction, evaluation and stable expression of scFv:sTRAIL fusion proteins in CHO-K1 cells[35]. Plasmid pEE14scFv:sTRAIL is based on a vector previously described[49]. Important features are the murine kappa light-chain leader peptide encoded upstream of 2 multiple cloning sites (MCSs) that are separated by a 26 residue in-frame linker sequence, and the glutamine synthetase selectable marker gene, which allows for amplified expression of the recombinant protein in production cell line CHO-K1. The vector exploits the strong CMV promoter to drive recombinant protein expression, while the leader peptide directs the fusion protein through the ER and Golgi complex, resulting in excretion of correctly folded fusion protein into the culture supernatant[35]. In the first MCS, a 45 bp DNA fragment encoding scFvCD7 derived from phagemid pCANTAB5E/scFv3A1F was

directionally inserted using unique SfiI and NotI restriction enzyme sites. The second MCS contains a PCR-truncated 593 bp DNA fragment encoding the extracellular domain of human TRAIL (sTRAIL).

Production and characterization of scFvCD7:sTRAIL

Fusion protein scFvCD7:sTRAIL was eukaryotically expressed in CHO-K1 cells essentially as described[35]. In short, CHO-K1 cells were transfected with plasmid pEE14scFvCD7:sTRAIL using Fugene-6 reagent (Roche). Stable transfectants with amplified expression were generated by the glutamine synthetase selection method[50]. Individual clones, obtained after single cell sorting using the Moflo high-speed cell sorter (Cytomation, Fort Collins, USA), were analyzed for stable and high expression of scFvCD7:sTRAIL in the absence of MSX selection reagent using a solid-phase sandwich TRAIL ELISA according to manufacturer's recommendations (Diaclone SAS, Besançon, France). The procedure identified CHO-K1 production cell line 10F1, which stably secreted scFvCD7:sTRAIL (7.3 µg/ml) into the medium. Large-scale production of scFvCD7:sTRAIL was performed using roller bottles (Greiner Bio-One, Frickenhausen, Germany) at 37°C in serum-free CHO-S SFM II suspension medium (Gibco, Life Technologies, Breda, The Netherlands) to a density of $5.0 \cdot 10^6$ cells/ml, after which supernatant was harvested (1500g, 10 min) and stored at -80°C until further use.

Size-Exclusion FPLC of scFvCD7:sTRAIL

Solution behaviour of scFvCD7:sTRAIL was analyzed by size-exclusion (SE) FPLC using a calibrated HiLoad 16/60 Superdex 200 Prep-grade column (Amersham Biosciences, Upsala, Sweden) with a bed volume of 120 ml; 5 ml supernatant derived from CHO-K1 cell line 10F1 was loaded onto the column, after which individual samples were collected at 3-min intervals. All samples were analyzed for their capacity to induce apoptosis using TRAIL-sensitive Jurkat cells. Furthermore, all samples were subjected to a sensitive TRAIL-specific ELISA to quantitate individual scFvCD7:sTRAIL content.

CD7-specific binding of scFvCD7:sTRAIL

CD7-specific binding of scFvCD7:sTRAIL was assessed using Ramos and Ramos.CD7 cells. In short, 1.0×10^6 cells were incubated with scFvCD7:sTRAIL in the presence or absence of CD7 MAb TH69 (5 µg/ml). CD7-specific binding of scFvCD7:sTRAIL to the cell surface of Ramos.CD7 cells was analyzed by flow cytometry using a PE-conjugated anti-TRAIL MAb (Diaclone SAS, Besancon, France). Incubations were carried out for 45 min at 0°C and were followed by two washes with serum free medium.

CD7-restricted induction of apoptosis by scFvCD7:sTRAIL

CD7-positive tumour cells were seeded at $0.5 \cdot 10^6$ cells/well in a 24-well plate and treated for 16 h with 100 ng/ml scFvCD7:sTRAIL (unless indicated otherwise), in the presence or absence of MAb TH69 (2 µg/ml) or MAb 2E5 (1 µg/ml). Induction of apoptosis was assessed using one of the following apoptosis assays: Assessment of apoptosis by monitoring exposure of phoshatidylserine; The early apoptotic feature of exposure of phosphatidyl serine on the outer membrane was analyzed by flow cytometry using an AnnexinV-FITC/PI kit (NeXins Research, Kattendijke, The Netherlands) according to manufacturer's instructions. Assessment of apoptosis by monitoring loss of mitochondrial membrane potential (Δψ); Δψ was analyzed by flow cytometry using the cell-permeant green-fluorescent lipophilic dye DiOC6 (Molecular Probes, Eugene, USA) actively taken up by intact mitochondria of living cells only. After treatment, cells were harvested by centrifugation (300xg; 5 min), incubated for 30 min at 37°C with fresh medium containing 0.1 µM DiOC6, washed once with PBS, and analyzed by FACS. Immunoblot analysis of caspase activation and PARP cleavage; Induction of apoptosis evidenced by activation of caspase-8 and caspase-3, and PARP cleavage, was assessed by immunoblot analysis using anti-caspase-8 (Cell signalling technology, Beverly, MA, USA), anti-active caspase-3 (BD biosciences, San Jose, CA, USA), and anti-PARP (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Appropriate secondary PO-conjugated antibodies were from DAKO Cytomation (Glostrup, Denmark). Cells were seeded in 6 well plates at a final concentration of $2.0 \cdot 10^6$ cells/ml and treated as indicated. Cell lysates were prepared and immunoblot analysis was performed essentially as described[35]. *Detection of apoptotic DNA fragmentation;* Apoptotic DNA-fragmentation was analyzed using MAb F7-26 (Alexis, Kordia Life Sciences) according to manufacturer's recommendations. MAb F7-26 specifically detects DNA fragmented by apoptosis without reactivity for otherwise fragmented double-stranded DNA[51].

Differential quantification of apoptosis in target and bystander cells during mixed culture experiments

For mixed culture experiments, differential cell membrane labelling of target and bystander cells was achieved using the Vibrant Multicolour Cell-Labelling kit (Molecular probes). Briefly, CD7-positive Jurkat cells were labelled with the red fluorescent dye DiI, while CD7-negative Ramos bystander tumour cells or 'innocent' bystander leukocytes were not labelled. Labelling was performed by incubation of Jurkat cells ($1.0 \cdot 10^6$ cells/ml in serum free medium) with 5 µM DiI (37°C, 5 min), followed by three washes with medium. DiI-labelled target and non-labelled bystander cells were mixed at the indicated ratios at a final concentration of $0.5 \cdot 10^6$ cells/well of a 24-well plate. After treatment, differential fluorescent characteristics of target cells and bystander cells were used to separately evaluate induction of apoptosis in both populations by Δψ or AnnexinV staining.

CD7-restricted apoptosis induction in patient-derived leukemic cells

Blood cells derived from T-ALL patients, containing >90% leukemic T-cells, were briefly cultured and subsequently analyzed for sensitivity to apoptosis induction by scFvCD7:sTRAIL. Cells were treated for 16 h with scFvCD7:sTRAIL (1 µg/ml) in the presence or absence of MAb TH69 or MAb 2E5. Alternatively, cells were treated with scFvCD7:sTRAIL or vincristin (10 ng/ml) alone or in combination. Apoptosis was assessed by AnnexinV/PI staining as described above.

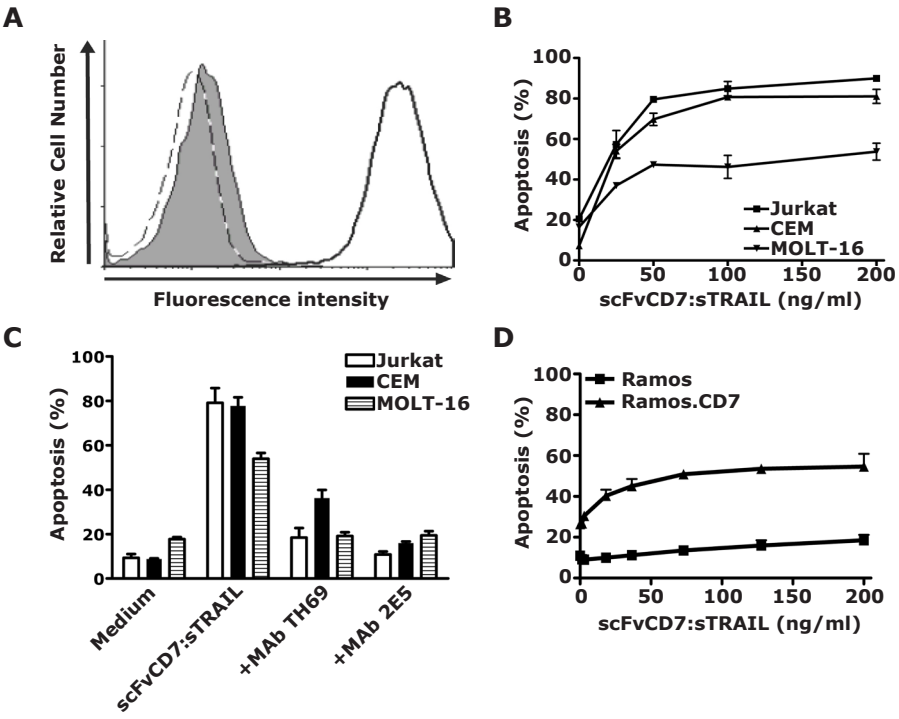

Fig.1. CD7-specific binding and apoptosis induction by scFvCD7:sTRAIL. A; Binding of scFvCD7:sTRAIL was analyzed by flow cytometry using CD7-negative Ramos and the transfectant cell line Ramos.CD7, ectopically over-expressing CD7. Ramos (solid fill) or Ramos.CD7 (solid line) were incubated with scFvCD7:sTRAIL. Additionally, Ramos.CD7 was pre-incubated with MAb TH69 followed by incubation with scFvCD7:sTRAIL (dashed line). **B;** CD7-positive T-ALL cell lines Jurkat, CEM, and MOLT-16 were treated for 16 h with increasing concentrations of scFvCD7:sTRAIL. **C;** Jurkat, CEM, and MOLT-16 were treated with scFvCD7:sTRAIL (100 ng/ml) in the presence or absence of MAb TH69 or TRAIL-neutralizing MAb 2E5. **D;** Ramos and Ramos.CD7 cells were treated for 16 h with increasing concentrations of scFvCD7:sTRAIL. In all of the above-described experiments, apoptosis was assessed by Δψ. Indicated values are mean + standard error of the mean of three independent experiments.

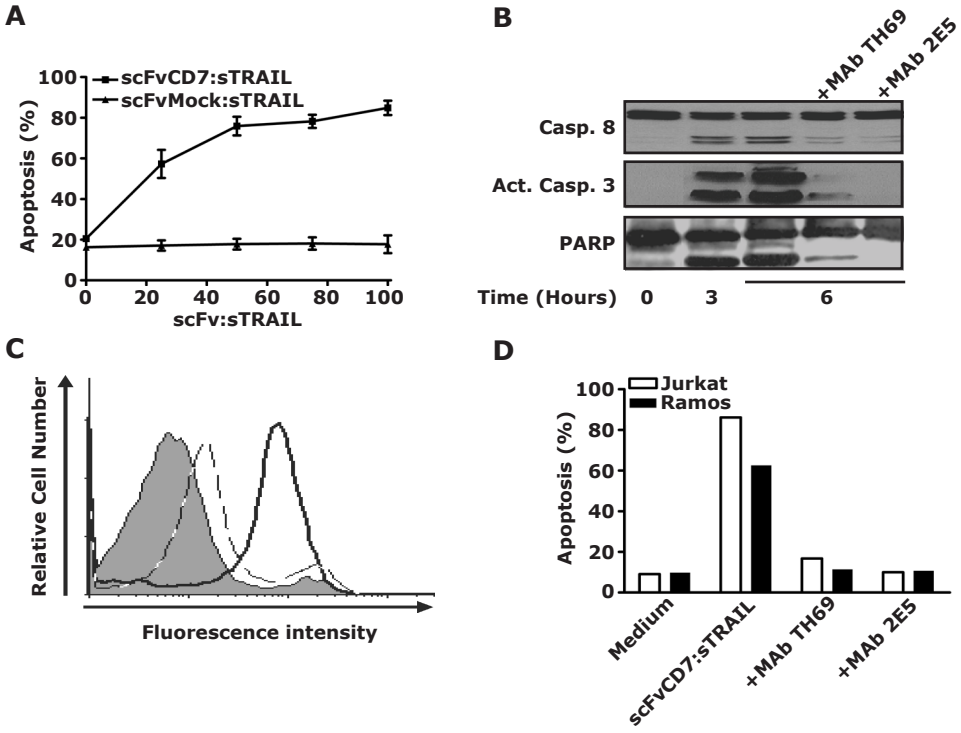

Fig.2. CD7-specific apoptosis induction by scFvCD7:sTRAIL. A; Jurkat cells were treated for 16 h with increasing concentrations of scFvCD7:sTRAIL or with MOCK-scFv:sTRAIL, containing an antibody fragment of irrelevant specificity, after which apoptosis was assessed by Δψ. Indicated values are representatives of three independent experiments **B;** Jurkat cells were treated with scFvCD7:sTRAIL (100 ng/ml) for the indicated time-points. For the 6 h incubation time, cells were additionally incubated with MAb TH69 or MAb 2E5. Cell lysates were assessed for the characteristic TRAIL-associated apoptotic features of caspase-8 activation, caspase-3 activation and PARP cleavage by immunoblot. **C:** Jurkat cells were treated for 24 h with 100 ng/ml scFvCD7:sTRAIL (solid line) in the presence or absence of MAb TH69 (dashed line). Apoptotic DNA fragmentation was assessed using MAb F7-26 as described in M&M section. Fluorescent intensity of conjugate control is shown as solid fill. **D;** Mixed cultures of Jurkat target cells and Ramos bystander cells (ratio 7:3) were treated for 16 h with scFvCD7:sTRAIL (300 ng/ml) in the presence or absence of MAb TH69 or MAb 2E5. Differential fluorescent labeling of the target and bystander population was used to separately assess apoptosis induction by Δψ. Indicated values are representatives of three independent experiments.

Results

Solution behavior of scFvCD7:sTRAIL

Supernatant of CHO-K1 clone 10F1 containing scFvCD7:sTRAIL was fractionated by SE-FPLC. Induction of apoptosis of the TRAIL-sensitive cell line Jurkat was restricted to individual samples collected after 97-114 min. The chromatographic mobility of

scFvCD7:sTRAIL corresponded to a molecular weight of approximately 160 kDa, in close agreement with the 154 kDa calculated for trimeric scFvCD7:sTRAIL. A sensitive TRAIL specific ELISA subsequently confirmed that only these fractions contained scFvCD7:sTRAIL, indicating that scFvCD7:sTRAIL was produced as homogenous trimers in the absence of high molecular weight aggregates (data not shown).

CD7-restricted binding and induction of apoptosis by scFvCD7:sTRAIL

Incubation of Ramos.CD7 cells with scFvCD7:sTRAIL, resulted in specific and abundant binding (Fig.1A, shaded peak). Binding to Ramos.CD7 cells was specifically inhibited by pre-incubation with CD7-competing MAb TH69 (Fig.1A, dashed line) to levels observed for CD7-negative Ramos cells (Fig.1A, solid fill) and, therefore, was CD7-specific. Binding of soluble scFvCD7:sTRAIL via its TRAIL domain to cell surface-expressed TRAIL receptors was also assessed by FACS. To this end, a MAb specific for the HA-tag present at the N-terminus of scFvCD7:sTRAIL was used. Binding of scFvCD7:sTRAIL to CD7-negative Ramos cells was barely detectable (data not shown).

Treatment of CD7-positive Jurkat, CEM, MOLT-16, and Ramos.CD7 cells with scFvCD7:sTRAIL for 16h resulted in pronounced induction of apoptosis at concentrations as low as 50 ng/ml (Fig.1B). Apoptosis was specifically inhibited by pre-treatment with MAb TH69 and co-treatment with TRAIL-neutralizing MAb 2E5 (Fig.1C). Parental Ramos cells were fully resistant to induction of apoptosis by scFvCD7:sTRAIL at all concentrations tested (Fig.1D). Treatment of CD7-positive Jurkat cells with a MOCK-scFv:sTRAIL fusion protein containing an scFv antibody fragment of irrelevant specificity, targeted at the carcinoma-associated antigen EGP2[35], did not induce apoptosis (Fig.2A). ScFvCD7:sTRAIL-mediated apoptosis was characterized by the activation of initiator caspase-8 and effector caspase-3, PARP cleavage (Fig.2B), and apoptotic DNA fragmentation (Fig.2C).

Induction of apoptosis in malignant bystander cells by scFvCD7:sTRAIL

In mixed culture experiments of CD7-positive target cells (Jurkat) and CD7-negative bystander cells (Ramos) (ratio 1:1), a potent anti-tumour bystander effect of up to 61% apoptosis was detected in Ramos bystander cells (Fig.2D). Apoptosis was abrogated in both target and bystander cells when treatment was preceded by incubation with CD7 MAb TH69.

scFvCD7:sTRAIL does not induce apoptosis in normal human leukocytes, activated T-cells and resting/TNF-α activated HUVEC

Incubation of freshly isolated leukocytes showed strong and specific binding of scFvCD7:sTRAIL to the cell surface of T-cells and NK-cells, but not to B-cells (Fig.3A).

Again, binding of scFvCD7:sTRAIL was specifically inhibited by treatment with MAb TH69 (Fig.3B). Treatment of freshly isolated leukocytes, containing both T-cells and NK cells, with scFvCD7:sTRAIL did not induce apoptosis in any of the blood cell types analyzed, even when treatment was prolonged to 8 days (Fig.3C). Also CD3/IL-2-activated T-cells were fully resistant to prolonged treatment with scFvCD7:sTRAIL (Fig.3D). Thus, the pro-apoptotic effect of scFvCD7:sTRAIL was restricted to CD7-positive malignant cells.

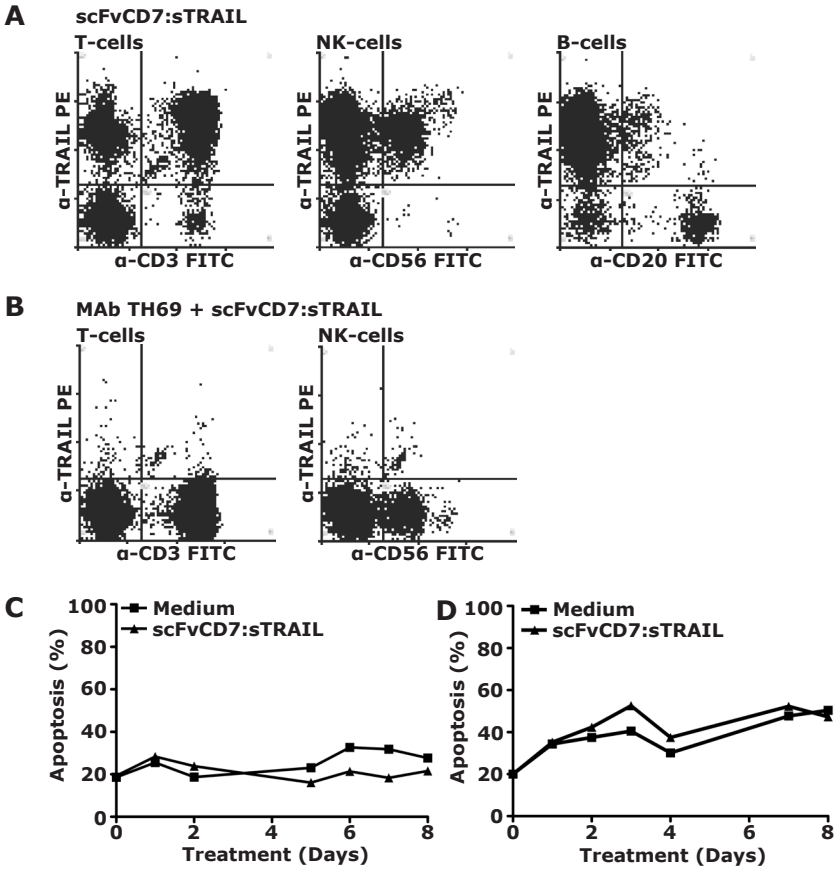

Fig.3. No apoptosis induction in normal human leukocytes and activated T-cells. A; Binding of scFvCD7:sTRAIL to freshly isolated leukocytes was analyzed by double staining using PE-conjugated anti-TRAIL and either a T-cell specific marker (anti-CD3 FITC), an NK-cell specific marker (anti-CD56 FITC), or a B-cell specific marker (anti-CD20 FITC). **B;** Leukocytes were co-incubated with scFvCD7:sTRAIL and MAb TH69, whereupon specific binding was determined. **C;** Resting PBLs and **D;** activated T-cells were subjected to treatment with scFvCD7:sTRAIL (1,5 μg/ml) for up to 8 days. Apoptosis induction was assessed by AnnexinV/PI staining.

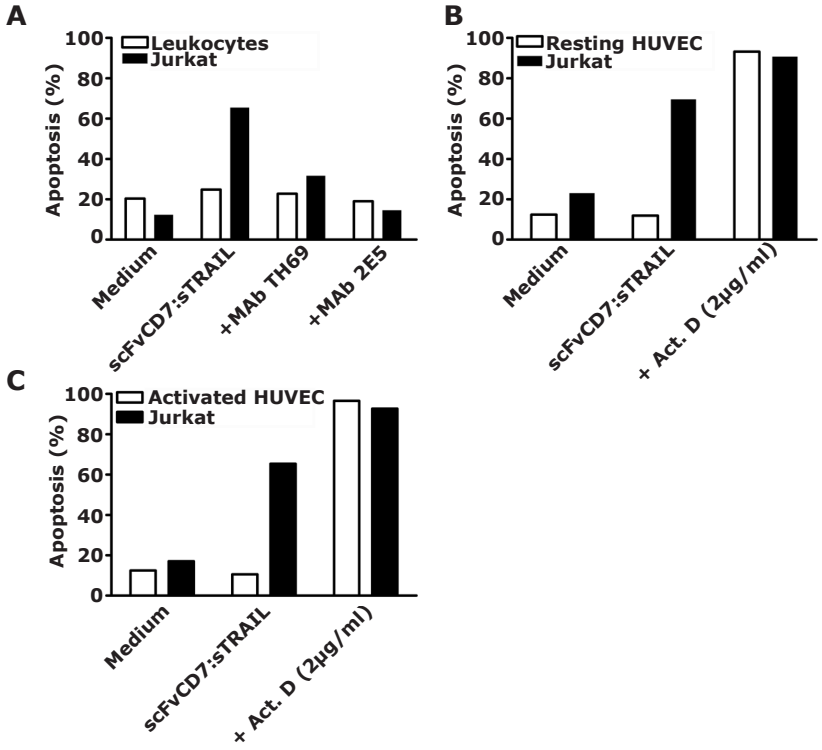

Fig.4. No apoptosis induction in normal human leukocytes and HUVEC. A; Isolated leukocytes were mixed at a ratio of 1:10 with DiI-labeled Jurkat cells. Mixed cultures were treated for 24h with scFvCD7:sTRAIL in the presence or absence of MAb TH69 or MAb 2E5. **B;** Resting HUVEC were mixed with Jurkat (ratio of 1:1) and subsequently treated with scFvCD7:sTRAIL or Actinomycin D (2 µg/ml) for 24 h. **C;** HUVEC were activated with TNF-α for 4 h and then treated with scFvCD7:sTRAIL or Actinomycin D (100 ng/ml) for 24 h. In all mixed culture experiments, the differential fluorescent labeling of Jurkat target and innocent bystander cells was used to separately evaluate apoptosis by AnnexinV staining. Indicated values are representatives of three independent experiments.

Next we assessed whether membrane bound scFvCD7:sTRAIL deposited on CD7-positive Jurkat cells could exert an 'innocent' bystander effect towards normal leukocytes by treatment of mixed cultures of Jurkat tumour cells and freshly isolated leukocytes (ratio 10:1) with scFvCD7:sTRAIL. Separate analysis of target cells and bystander cells showed no increase in apoptosis in leukocyte bystander cells, while induction of apoptosis in Jurkat target cells reached up to 65% (Fig.4A).

To assess the apoptotic activity of scFvCD7:sTRAIL deposited on CD7-positive Jurkat cells towards innocent vascular endothelial bystander cells, Jurkat cells were co-cultured with resting - or TNF-α activated HUVEC cells at a ratio of 4:1. No increase in apoptosis

was observed, neither in resting (Fig.4B) nor in activated HUVEC cells (Fig.4C), while induction of apoptosis in Jurkat cells reached up to 69%.

scFvCD7:sTRAIL induces more potent apoptosis than the immunotoxin scFvCD7:ETA

The pro-apoptotic activities of scFvCD7:sTRAIL and the immunotoxin scFvCD7:ETA were compared by treating Jurkat and CEM cells with equimolar concentrations of either fusion protein. After treatment for 24 h the apoptotic activity of scFvCD7:sTRAIL was clearly stronger than that of scFvCD7:ETA (Fig.5A). Stronger induction of apoptosis by scFvCD7:sTRAIL was maintained when treatment was prolonged to 72 h (data not shown).

As stated above, both freshly isolated leukocytes and activated-T cells were resistant to prolonged treatment with scFvCD7:sTRAIL. However, when resting leukocytes or activated T-cells were treated with equimolar amounts of scFvCD7:ETA, a marked induction of apoptosis of up to 46% after 72 h was observed in resting leukocytes (Fig.5C), while approximately 76% apoptosis induction was observed in activated T-cells (Fig.5D).

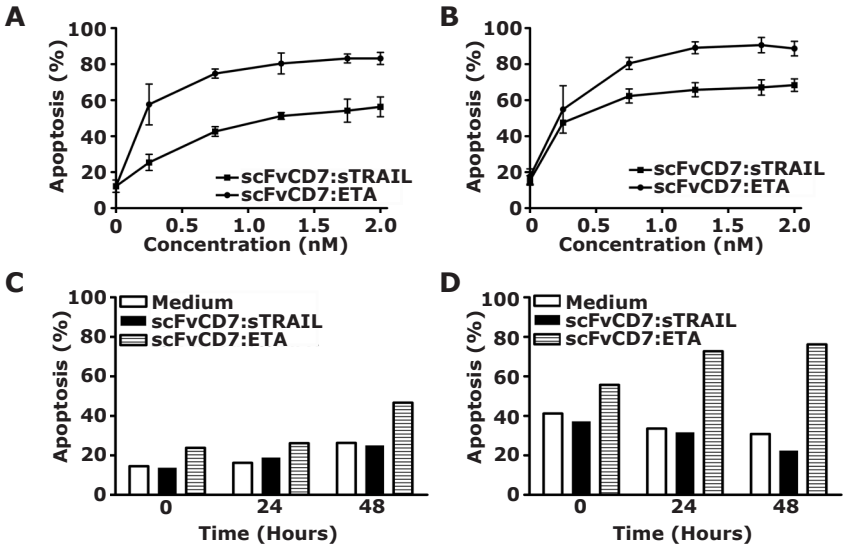

Fig.5. Activity of scFvCD7:sTRAIL compared to scFvCD7:ETA A; CEM and **B;** Jurkat cells were treated for 24h with increasing equimolar concentrations of scFvCD7:sTRAIL and scFvCD7:ETA. Indicated values are mean + standard error of the mean of three independent experiments **C;** resting leukocytes and **D;** activated T-cells were treated for 24, 48 and 72 h with an equimolar concentration (1.75 nM) of either scFvCD7:sTRAIL or scFvCD7:ETA. Indicated values are representatives of three independent experiments. Apoptosis induction was assessed by AnnexinV/PI staining.

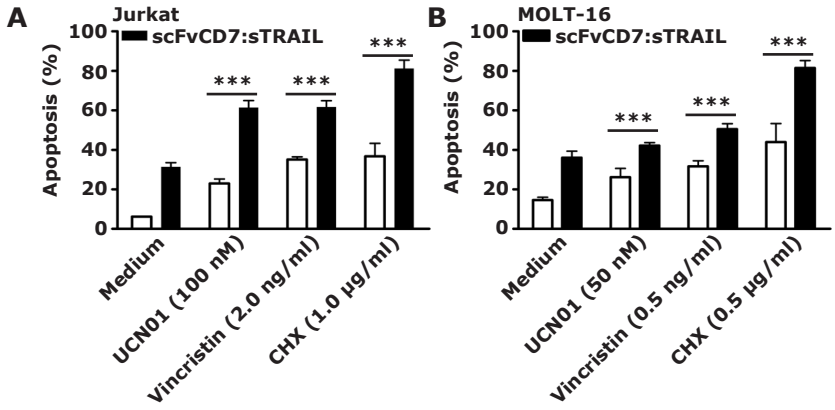

Fig.6. Potentiation of scFvCD7:sTRAIL treatment by several classes of chemotherapeutics. A; Jurkat and **B;** MOLT-16 were treated for 16 h either alone or simultaneously with scFvCD7:sTRAIL (15 ng/ml) and Vincristin, UCN01, and CHX, at the concentrations indicated. Apoptosis induction was assessed by Δψ.

Augmentation of scFvCD7:sTRAIL activity by several classes of chemotherapeutics

Jurkat and MOLT-16 cells were treated with scFvCD7:sTRAIL in the presence or absence of established - and recently developed therapeutics (Fig.6A and B, respectively). Co-treatment with scFvCD7:sTRAIL and the microtubule inhibitor Vincristin resulted in significant additive induction of apoptosis of 27% and 19% in Jurkat and MOLT-16 cells, respectively. Co-treatment with the protein synthesis inhibitor CHX resulted in an additive induction of apoptosis of 44.5% and 37.5%, while co-treatment with the staurosporin analog UCN01 increased the induction of apoptosis with 39% and 15% in Jurkat and MOLT-16 cells, respectively. On normal human PBLs, activated T-cells, and HUVECs, combination treatment with either of the drugs and scFvCD7:sTRAIL did not result in increased apoptosis (data not shown).

Synergistic induction of apoptosis in patient-derived T-ALL cells by scFvCD7:sTRAIL and vincristin

Blood cells freshly derived from three T-ALL patients were subjected to treatment with scFvCD7:sTRAIL (1 µg/ml) for 16 h, after which induction of apoptosis was visualized by AnnexinV/PI staining (Fig.7A). In two out of three T-ALL patients, treatment with scFvCD7:sTRAIL markedly induced apoptosis (25% and 16%, respectively), whereas one patient sample was resistant to treatment. Apoptosis induction by scFvCD7:sTRAIL was specifically inhibited by pre-incubation with CD7 MAb TH69 or TRAIL-neutralizing MAb 2E5 (Fig.7B). Combination treatment of primary T-ALL patient material with scFvCD7:sTRAIL and vincristin resulted in over 50% apoptosis (Fig.7C), whereas single

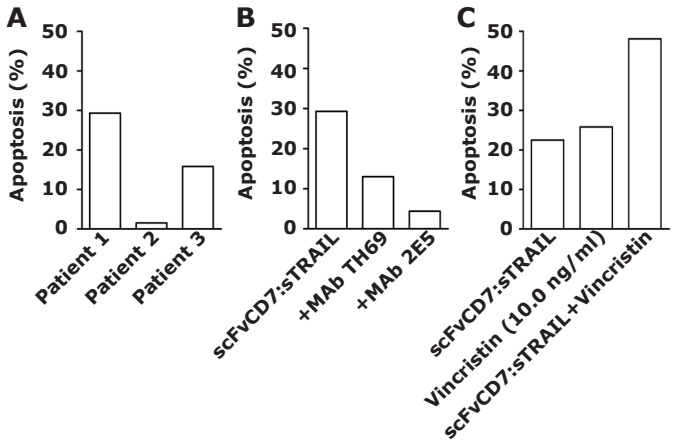

Fig.5. Apoptosis induction by scFvCD7:sTRAIL in patient derived T-ALL cells. A: Blood cells directly derived from T-ALL patients, containing >90% leukemic T-cells, were subjected to treatment with scFvCD7:sTRAIL (1 µg/ml). **B;** Primary T-ALL patient material was subjected to scFvCD7:sTRAIL (1 µg/ml) in the presence of MAb TH69 or MAb 2E5. **C;** T-ALL patient material was subjected to single agent treatment with scFvCD7:sTRAIL, vincristin (10 ng/ml), or to combination treatment with scFvCD7:sTRAIL and vincristin. In all experiments, apoptosis was assessed by AnnexinV/PI staining.

agent treatment induced approximately 20% apoptosis. Induction of apoptosis was inhibited when treatment was performed in the presence of CD7 MAb TH69 or TRAIL-neutralizing MAb 2E5 (data not shown).

Discussion

In recent years an increased understanding of pathogenic mechanisms has provided new targets and strategies for anti-leukemic therapy. These range from novel chemotherapeutic agents, therapeutic antibodies, bispecific antibodies, immunotoxins and radioimmunoconjugates, to targeted therapy with small molecules interfering with key cellular components such as tyrosine kinases. Here we describe a novel promising approach for the therapy of CD7-positive T-cell leukemia by induction of target antigen-restricted apoptosis using a recombinant scFvCD7:sTRAIL fusion protein with specificity for CD7.

Our experiments demonstrate specific binding of scFvCD7:sTRAIL to the cell surface of CD7-positive cells only. Binding of scFvCD7:sTRAIL to TRAIL-receptors on CD7-negative tumour cells via its TRAIL domains was often below detectable levels, which might be explained by the fact that polypeptide ligands, such as TRAIL, have typical fast-on/fast off binding rates. In contrast, antibody fragments, such as scFvs, usually retain the fast-on/slow-off rates typical for antibody-mediated binding. Stable trimeric scFvCD7:sTRAIL contains 3 identical scFv domains, which potentially enhances binding

to CD7-positive cells by the associated avidity effect. Enhanced avidity has been shown to be beneficial for *in vivo* tumour targeting in many antibody-based therapeutic strategies[52,53]. Moreover, the CD7 target antigen was selected for its specific and abundant surface expression on human T-cell leukemia and lymphoma. Although not examined in detail here, we have indications that the number of CD7 molecules on the surface of T-ALL cells greatly exceeds that of TRAIL receptors. Taken together, these arguments explain why scFvCD7:sTRAIL predominantly binds to target cells via its scFv domain.

CD7-selective binding increases the local concentration on the target cell surface which allows the sTRAIL domain of scFvCD7:sTRAIL to bind to proximal TRAIL receptors more frequently, thereby, enhancing the pro-apoptotic signalling. As previously shown, target antigen-bound scFv:sTRAIL acquires TRAIL-receptor activating properties resembling that of native memTRAIL. Similarly, scFvCD7:sTRAIL can fully activate not only TRAIL-R1 but also TRAIL-R2 upon specific CD7-mediated immobilization to the cell surface of targeted cells. Treatment of a series of CD7-positive T-ALL cell lines with scFvCD7:sTRAIL potently induced apoptosis, evidenced by activation of caspase-8, caspase-3, PARP cleavage, and apoptotic DNA fragmentation. When treatment was performed in the presence of CD7-blocking MAb TH69, apoptosis was strongly inhibited, which clearly demonstrated that scFvCD7:sTRAIL performed its pro-apoptotic action in an antigen-restricted manner. FACS analysis provided evidence that selective binding to CD7 led to the exclusive deposition of scFvCD7:sTRAIL on the cell surface of targeted cells. As a consequence, a surplus of CD7-bound scFvCD7:sTRAIL becomes available on the cell surface for binding and crosslinking of agonistic TRAIL receptors on neighbouring tumour cells. When neighbouring cells are also CD7-positive, a strong reciprocal 'fratricide' apoptosis of tumour cells is induced.

The function of CD7 and its possible ligands are still largely unknown. Recent reports indicate that Galectin-1 mediated crosslinking of CD7 induces apoptosis in activated T-cells and T-ALL cells[54]. We asked whether crosslinking of CD7 on T-ALL cells by soluble trimeric scFvCD7:sTRAIL would be sufficient to induce apoptosis. Therefore, T-ALL cells were treated with scFvCD7:sTRAIL in the presence of a TRAIL-neutralizing MAb. As a result, apoptotic activity was almost completely abrogated, demonstrating that apoptosis by scFvCD7:sTRAIL was pre-dominantly TRAIL-mediated.

As CD7 is expressed on a large subset of normal human T-cells and NK-cells, the potential for unwanted apoptosis by CD7-specific binding of scFvCD7:sTRAIL was examined in resting and activated normal blood cells. Both resting leukocytes and activated T-cells were resistant to treatment with scFvCD7:sTRAIL for up to 8 days, with no increase in apoptosis compared to control experiments. The striking preferential pro-apoptotic activity of TRAIL and TRAIL-fusion proteins for tumour cells over normal cells has been reported by other authors, but the underlying molecular mechanism remains unclear.

Subsequently, the possibility of a so-called 'innocent bystander' effect of cell surface

deposition of scFvCD7:sTRAIL on CD7-positive leukemia cells towards neighbouring normal blood cells or HUVEC cells was assessed. In an *in vitro* model, Jurkat cells were co-cultured with normal human leukocytes. Separate analysis of these leukocytes showed no increased apoptosis in leukocyte bystander cells. Similarly, no innocent bystander apoptosis was observed towards resting and TNF-α activated HUVEC cells.

Bystander activity towards target antigen-negative tumour cells may be of great value in cases where heterogeneous or lost target antigen expression allows tumour cells to escape from therapy. Antibody-based therapy of leukemia has been associated with target antigen-negative recurrences after treatment with Rituximab in B-cell lymphoma[55,56] and CAMPATH-1H in T-cell prolymphocytic leukemia[57]. The bystander effect of scFvCD7:sTRAIL is based on the principle that targeted tumour cells are not only eliminated, but are also exploited to crosslink agonistic TRAIL receptors on neighbouring tumour cells lacking the target antigen. Recently, we reported on an exceptionally potent anti-tumour bystander effect of an analogous scFv:sTRAIL fusion protein with specificity for a carcinoma-associated cell surface antigen[58]. In the current study we made similar observations when mixed cultures of CD7-negative Ramos cells and CD7-positive Jurkat cells were treated with scFvCD7:sTRAIL. Potent bystander apoptosis towards the CD7-negative Ramos cells was observed, which was fully dependent on CD7-specific binding of scFvCD7:sTRAIL to the surface of Jurkat cells.

Currently, various MAb-toxin conjugates and a small number of scFv:toxin fusion proteins are being evaluated for their therapeutic application in human cancer. However, targeted therapy using toxin-based conjugates has imperative drawbacks. In order to specifically kill a target cell, binding of a toxin-based molecule must be followed by efficient cellular internalization. Subsequently, the toxin must be delivered to the appropriate intracellular compartment in order to exert its full cytotoxic effect. These features limit the choice of target antigens on malignant cells to those known to rapidly internalize after binding. Importantly, most if not all currently used toxins are equally toxic to both normal and malignant cells. Therefore, the safety and efficacy of MAb-toxins and scFv:toxins solely relies on the tumour-selectivity of the antibody used. In contrast, targeted therapy using scFvCD7:sTRAIL does not require internalization, intracellular enzymatic conversion or trafficking to exert its pro-apoptotic effect. Furthermore, TRAIL was previously shown to have a surprising intrinsic tumour-selective activity. Consequently, the safety and efficacy of scFv:sTRAIL fusion proteins is determined by both the tumour-selective activity of TRAIL and the tumour-selectivity of the antibody fragment used. Moreover, a broad array of cell surface molecules can be used as target antigens, even those that are not strictly cancer-associated such as CD7, which is abundantly expressed on normal T-cells and NK cells.

The tumour selectivity of our TRAIL-based fusion protein scFvCD7:sTRAIL was directly

compared with the ETA-based immunotoxin scFvCD7:ETA. At equimolar concentrations, scFvCD7:sTRAIL was significantly more potent than scFvCD7:ETA. Furthermore, in contrast to treatment with scFvCD7:sTRAIL, resting peripheral blood lymphocytes were sensitive to treatment with scFvCD7:ETA. After treatment for 72 h, a marked increase in apoptosis was noted. Activated normal T-cells showed an even more pronounced apoptotic response to scFvCD7:ETA.

Recently, pro-apoptotic effects of certain sTRAIL preparations towards HUVEC and other normal cell types were reported[59-63]. Differences reported for TRAIL–related toxicity might be due to solution behaviour of the various sTRAIL preparations used. It was shown that prokaryotically produced HIS-tagged sTRAIL preparations can contain high molecular weight aggregates that cause toxicity towards hepatocytes[32]. We chose to produce scFvCD7:sTRAIL using CHO-K1 cells, an established industry-grade eukaryotic production system for recombinant therapeutic protein drugs. Previously, this system was shown to produce homogenous and biologically active scFv:sTRAIL trimers in the absence of high molecular weight aggregates[35]. In our experiments, even cell-surface bound scFvCD7:sTRAIL produced no apoptosis in neighbouring normal cells. Apparently, crosslinking of agonistic TRAIL receptors by cell surface-bound TRAIL significantly differs from receptor crosslinking by aggregated TRAIL species. Nevertheless, further *in vivo* research is needed to exclude unwanted apoptotic activity of scFvCD7:sTRAIL towards normal human cells and tissues.

ScFvCD7:sTRAIL treatment was combined with a number of chemotherapeutic agents to evaluate whether apoptotic activity could be significantly enhanced. Treatment with scFvCD7:sTRAIL and the microtubule inhibitor vincristin, a chemotherapeutic agent long part of clinical practice for T-ALL, strongly enhanced apoptosis. Co-treatment with PKC/cyclin inhibitor UCN01, a recently developed anti-leukemic agent, also significantly enhanced apoptosis. Additionally, inhibition of protein synthesis by CHX strongly potentiated apoptosis. Most likely other cytotoxic regimes can be identified that significantly enhance the target-cell restricted apoptotic activity of scFvCD7:sTRAIL to further improve its anti-leukemic effect with no or reduced overlapping toxicities.

Previous reports on targeted leukemia therapy indicated that leukemic cells freshly derived from patients responded more poorly to treatment when compared to the leukemia-derived cell lines. When blood cells derived from T-ALL patients, containing >90% T-ALL cells, were treated with scFvCD7:sTRAIL a marked CD7-restricted and TRAIL-mediated induction of apoptosis was observed in two out of three patients. When treatment was performed in the presence of vincristin, induction of apoptosis was strongly enhanced in a synergistic manner.

In conclusion, scFvCD7:sTRAIL is a representative of a novel class of immunotherapeutic molecules, which acts by inducing apoptosis in an antigen-restricted manner, but avoids

undesirable side-effects of known immunotoxins. The potent and highly selective anti-leukemic activity of scFvCD7:sTRAIL, either alone or in combination with chemotherapeutic agents, holds great promise for the treatment of human T-cell tumours.

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CD7-restricted activation of Fas-mediated apoptosis: a novel therapeutic approach for acute T-cell leukemia.

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chapter 7

Abstract

Agonistic anti-FAS antibodies and multimeric recombinant FASL preparations show highly potent anti-leukemia activity, but are not suitable for clinical application due to unacceptable systemic toxicity. Consequently, new anti-leukemia strategies based on FAS activation have to meet the criterion of strictly localized action at the tumour cell surface. Recent insight into the FASL/FAS system has revealed that soluble homotrimeric FASL (sFASL) is in fact non-toxic to normal cells, but also that it lacks tumoricidal activity. We report on a novel fusion protein, designated scFvCD7:sFASL, designed to have leukemia-restricted activity. ScFvCD7:sFASL consists of sFASL genetically linked to a high affinity scFv antibody fragment specific for the T-cell leukemia-associated antigen CD7. Soluble homotrimeric scFvCD7:sFASL is inactive and acquires tumoricidal activity only after specific binding to tumour cell surface-expressed CD7. Treatment of T-ALL cell lines and patient-derived T-ALL, PTCL, and CD7-positive AML cells with homotrimeric scFvCD7:sFASL revealed potent and CD7-restricted apoptosis induction that could be augmented by various conventional drugs, farnesyl transferase inhibitor L-744,832, and proteasome inhibitor Velcade. Importantly, identical treatment did not affect normal human PBLs and endothelial cells, with only moderate apoptosis induction in IL-2/CD3-activated T-cells. The leukemia-restricted activation of FAS by homotrimeric scFvCD7:sFASL revitalizes the applicability of FAS signalling in leukemia therapy.

Introduction

Despite advances in T-cell leukemia therapy, only a minority of patients achieves long term tumour-free survival with conventional chemotherapy at the cost of significant and often irreversible toxic side effects.[1] Therefore, new therapeutic approaches with enhanced tumour selectivity and more favourable toxicity profiles are urgently needed. Several promising targeted approaches have been developed, including naked antibodies[2,3], immunotoxins[4,5], and various cancer-selective small inhibitory molecules[6,7]. Furthermore, certain members of the TNF-superfamily show promising pro-apoptotic activity towards various human leukemias and lymphomas.

FASL, a prominent member of the TNF-superfamily, shows superior anti-leukemia activity. FASL is present on lymphocytes and monocytes/macrophages as a type II transmembrane protein, hereafter referred to as memFASL. FAS, the cognate receptor for FASL, belongs to the growing family of transmembrane proteins known as death receptors. Death receptors can detect the presence of specific extracellular death signals and rapidly trigger cellular destruction by apoptosis. FAS expression at the cell surface is observed in biopsies and cell lines derived from a variety of tumours. Moreover, the anti-tumoral effects of various chemotherapeutic drugs have been attributed partly to p53-mediated up-regulation of FAS and FASL[8-12]. FAS signalling is also known to be a key element in the effector phase

of a CTL response against tumour cells.

Like other members of the TNF-superfamily, the extra-cellular domain of FASL can be proteolytically cleaved into a soluble homotrimeric form[13-15], hereafter referred to as sFASL.

Early attempts to exploit FAS agonists such as anti-FAS antibodies and multimeric recombinant FASL preparations for therapy revealed extremely potent tumoricidal effects towards isolated primary tumour cells and cell lines[13,16-19]. However, *in vivo* application of most FAS antagonists was associated with acute lethality in mice[20-22], thereby excluding therapeutic evaluation in humans. Nevertheless, the principal feasibility of therapeutic FAS activation in cancer therapy was clearly demonstrated in mice that lack a functional FASL/FAS system (lpr/gld mice)[23] and by treatment of xenografted tumours with human FAS-specific antibodies[24].

Recent studies have revealed that certain recombinant sFASL preparations contain oligomeric, multimeric, and even aggregated sFASL forms and that these forms are responsible for the observed systemic toxicity[25]. In contrast, homotrimeric sFASL is not toxic to normal cells and may even antagonize the function of membrane bound FASL[25-27]. Importantly, homotrimeric sFASL also lacks tumoricidal activity. However, inactive homotrimeric sFASL can rapidly be re-activated by applying secondary crosslinking antibodies.

Recently, we demonstrated that the leukemia selectivity of homotrimeric TRAIL, another TNF-superfamily-member, can be strongly enhanced by genetically fusing it to a CD7-selective antibody fragment[28]. Human CD7 is a lineage-specific antigen that is highly expressed on acute T cell leukemia and ~10% of acute myeloid leukemia[29-32]. The function of CD7 is not yet fully understood. In normal cells CD7 expression is limited to T- and myeloid cells in early hematopoietic cell ontogeny, thymocytes, NK cells, and to a distinct subset of peripheral blood T-cells[33-37]. Human CD7 has been used for the targeted delivery of several MAb-toxin conjugates in both pre-clinical studies and clinical trials[4, 5,38,39].

Here we report on a novel homotrimeric sFASL fusion protein, designated scFvCD7:sFASL with enhanced and leukemia-restricted activity towards T-ALL cell lines and patient-derived T-ALL, PTCL, and CD7-positive AML cancer cells. We provide evidence that homotrimeric scFvCD7:sFASL is bioactive only after specific binding to cell surface-expressed CD7 with no toxicity towards CD7-negative cells and only moderate activity towards IL-2/CD3-activated CD7-positive T-cells.

Materials & Methods

Monoclonal antibodies and scFv antibody fragment

MAb TH69 is a murine IgG1 with specificity for human CD7[3] and was kindly provided by Prof. Dr. Martin Gramatzki, Division of Stem Cell and Immunotherapy, 2nd

Medical Department, University Clinic Schleswig-Holstein, Kiel, Germany. Phagemid pCANTAB5E/scFv3A1F encoding anti-CD7 antibody fragment 3A1F[40] was kindly provided by Dr. Chris Pennell, Department of Laboratory Medicine and Pathology, University of Minnesota. MAb TH69 and scFv-3A1F compete for binding to the same or overlapping epitope on the extracellular domain of human CD7. FASL-neutralizing MAb Alf2.1 was purchased from Sigma (Sigma-Aldrich Chemie B.V. Zwijndrecht, The Netherlands).

Chemotherapeutics

The cytostatic drugs used are Vincristin (stock; 1 mg/ml in PBS), Amsacrine (stock; 1mM in PBS), and Actinomycin D (stock; 2 mg/ml in ethanol). Farnesyl Transferase inhibitor L-744,832 was purchased from Merck (Darmstadt, Germany) and was dissolved at 10 mM in DMSO. The proteasome inhibitor Velcade (Millennium pharmaceuticals, Cambridge, MA) was dissolved at 10mM in dH_2O. All final concentrations were prepared by serial dilutions in serum free medium.

Cell lines

Human CD7-positive T-ALL cell lines Jurkat, CEM, and the CD7-negative human B-cell lymphoma cell lines Ramos and Raji were purchased from the ATCC (Manassas, USA). T-cell lines MOLT-16, HuT-78 were a kind gift of Prof. Dr. Martin Gramatzki. A CD7-positive transfectant of the Ramos cell line (Ramos.CD7) was generated as previously described[28]. All cell lines were cultured in RPMI (Cambrex, New Jersey, New Hampshire, USA) supplemented with 13% FCS, at 37ºC in humidified 5% CO_2 atmosphere.

Leukocytes, PBLs, activated T-cells, and HUVECs

Leukocytes were isolated from whole blood of healthy donors by a standard Ammonium Chloride method[41]. Peripheral blood lymphocytes (PBLs) were isolated from whole blood of healthy donors by standard density gradient centrifugation procedures (Lymphoprep, Axis-Shield PoC As., Oslo, Norway). Freshly isolated PBLs were resuspended at $2{\cdot}10^6$ cell/ml in RPMI, supplemented with 10% Human Pooled Serum. Activated T-cells were obtained by incubation of freshly isolated PBLs with anti-CD3 MAb WT32 (0.5 μg/ml) for 72 h, followed by IL-2 stimulation (100 ng/ml) for 48 h. HUVECs were isolated as previously described[42]. HUVEC cells were used before culture passage number four and, for experiments, were pre-cultured in 6 well plates at 60% confluency. HUVEC cells were activated with TNF-α or IFN-γ at a final concentration of 100ng/ml.

Construction of scFvCD7:sFASL

Previously, we constructed the eukaryotic expression plasmid pEE14scFv:sTRAIL for the rapid construction, evaluation and stable expression of scFv:sTRAIL fusion proteins in

CHO-K1 cells[43]. Important features of this vector are the presence of the murine kappa light-chain leader peptide encoded upstream of 2 multiple cloning sites (MCSs) that are separated by a 26 residue in-frame linker sequence, and the glutamine synthetase selectable marker gene, which allows for amplified expression of the recombinant protein in the established industrial production cell line CHO-K1. The vector exploits the strong CMV promoter to drive recombinant protein expression, while the leader peptide directs secretion of the fusion protein into the culture supernatant. In the first MCS, a 745 bp DNA fragment encoding anti-CD7 scFv3A1F derived from Phagemid pCANTAB5E/scFv3A1F was directionally inserted using the unique *Sfi*I and *Not*I restriction enzyme sites. In the second MCS, sTRAIL encoding cDNA was swapped for a PCR-truncated 539 bp DNA fragment encoding the extracellular domain of human sFASL using restriction enzymes *Xho*I and *Hin*dIII and standard DNA manipulation procedures. FASL cDNA truncation was performed by PCR using proofread DNA polymerase according to standard protocol using primers: T1: 5'-ATCCTCGAGTCTAGTGGGAGCGGATCTACCAGCCAGATGCACACA-3' (*Xho*I site is underlined) and T2: 5'-CCCAAGCTTTGCTTCTCTTAGAGCTTATATAAG-3' (*Hin*dIII site is underlined).

Production of scFvCD7:sFASL

ScFvCD7:sFASL was expressed in CHO-K1 cells with the glutamine synthetase selection/amplification system essentially as described previously[43]. Briefly, CHO-K1 cells were transfected with pEE14scFvCD7:sFASL using Fugene-6 reagent (Roche Diagnostics, Almere, The Netherlands). Stable transfectants with amplified expression were isolated and single cell sorted with a high-speed cell sorter (Cytomation, Fort Collins, USA). Individual clones were assessed for stable and high secretion of scFvCD7:sFASL in the absence of the MSX selection reagent by a FASL ELISA according to manufacturer's recommendations (Alexis, 10P's BVBA, Breda, The Netherlands). This procedure identified CHO-K1 production cell line 100B2, which stably secreted scFvCD7:sFASL (1.34 µg/ml) into the medium. ScFvCD7:sFASL containing supernatant was harvested (10,000xg; 10 min) and stored at -80°C until use.

Solution behaviour of scFvCD7:sFASL

Size-exclusion FPLC: The solution behaviour of scFvCD7:sFASL was analyzed by size-exclusion (SE) FPLC with a calibrated HiLoad 16/60 Superdex 200 Prep-grade column (Amersham Biosciences, Uppsala, Sweden) of a bed volume of 120 ml; 5 ml supernatant derived from CHO-K1 cell line 100B2 was loaded onto the column, after which individual samples were collected at 3-min intervals. Individual samples were analyzed for their capacity to induce apoptosis in CD7-positive FASL-sensitive MOLT-16 cells.

CD7-specific binding of scFvCD7:sFASL

CD7-specific binding of scFvCD7:sFASL was assessed by incubation of $1.0 \cdot 10^6$ CEM cells with scFvCD7:sFASL containing medium (1.34 µg/ml) in the presence or absence of CD7-blocking MAb TH69 (5 µg/ml). CD7-specific binding was analyzed by flow cytometry with PE-conjugated anti-FASL MAb (Diaclone SAS, Besancon, France). Incubations were carried out for 45 min at 0°C and were followed by two washes with serum free medium.

CD7-restricted apoptosis induction by scFvCD7:sFASL

Tumour cells were seeded at $0.25 \cdot 10^6$ cells/well in a 48-well plate and treated for 16 h with the indicated concentrations of scFvCD7:sFASL in the presence or absence of MAb TH69 (5 µg/ml) or MAb ALF2.1 (1 µg/ml). Apoptosis was assessed by one of the below-described assays. Percentage of specific killing was calculated using the following formula: (experimental apoptosis - spontaneous apoptosis)/(100 - spontaneous apoptosis)x100%. Assays employed to assess apoptosis: PS exposure to the outer cell membrane; flow cytometric analysis of exposure of phosphatidyl serine (PS) on the outer membrane with an AnnexinV-FITC/PI kit (NeXins Research, Kattendijke, The Netherlands) according to manufacturer's instructions. Loss of mitochondrial membrane potential ($\Delta\psi$); $\Delta\psi$ was analyzed with the cell-permeant green-fluorescent lipophilic dye DiOC6 (Molecular Probes, Eugene, USA). After treatment, cells were harvested by centrifugation (300xg; 5 min), incubated for 20 min at 37°C with 0.1 µM DiOC6 in fresh medium, washed once with PBS, and analyzed by flow cytometry. Fluorescence microscopy of activated caspase-3; After treatment, cells were spotted on microscope slides and fixed in acetone. Active caspase 3 staining was performed with MAb 5A1 (Cell Signalling) and secondary FITC-conjugated antibody (DAKO). DAPI was used to stain all nuclei. Specific staining was evaluated using a Quantimed 600S fluorescence microscope (Leica Camera Ag, Solms, Germany). Detection of apoptotic DNA fragmentation; DNA-fragmentation was analyzed using MAb F7-26 (Alexis) according to manufacturer's recommendations. MAb F7-26 specifically detects DNA fragmented by apoptosis without reactivity for otherwise fragmented double-stranded DNA[44].

Differential quantification of apoptosis in target and bystander cells in mixed culture experiments

For mixed culture experiments, CD7-positive target cells were labeled with the red fluorescent dye DiI (Molecular probes). Briefly, cells ($1.0 \cdot 10^6$ ml^{-1}) were incubated for 5 min at 37°C in serum free medium containing 5 µM DiI, followed by three washes with standard medium. DiI-labeled target and non-labeled bystander cells were mixed at indicated ratios with a final cell concentration of $0.5 \cdot 10^6$ cells/well in a 48-well plate. After treatment, differential fluorescent characteristics of target cells and bystander cells were

used to separately evaluate apoptosis by PS exposure to the outer cell membrane or by Δψ as described above.

Additive induction of apoptosis by scFvCD7:sFASL, chemotherapeutics and small inhibitory molecules

Additive apoptotic effects of treatment of cells with scFvCD7:sFASL and various chemotherapeutics or small inhibitory molecules was determined using the cooperativity index (CI), in which the sum of apoptosis induced by single-agent treatment is divided by apoptosis induced by combination-treatment. CI<1, treatment was termed synergistic; CI=1, treatment was termed additive; CI>1, treatment was termed antagonistic.

CD7-restricted apoptosis induction in patient-derived leukemic cells

Blood cells derived from four individual T-ALL patients (patients #1 to #4), one PTCL patient (patient #5), and one CD7-positive AML patient (patient #6), were treated for 16 h with scFvCD7:sFASL (150 ng/ml) in the presence or absence of MAb TH69, where indicated. Additionally, blood cells derived from patient #6 (AML) were co-treated with either Vincristin or Amsacrine. Apoptosis induction was assessed by PS exposure to the outer cell membrane and staining for active caspase 3 as described above.

Results

Fractionation and stability of homotrimeric scFvCD7:sFASL

Fractionation of crude supernatant containing scFvCD7:sFASL by SE-FPLC and subsequent assessment of the apoptotic activity of each separate fraction revealed two peaks of apoptotic activity (Fig.1A). One peak corresponded to a molecular weight (MW) of approximately 160 kDa, which closely resembles that of the calculated MW of 158 kDa for homotrimeric scFvCD7:sFASL. Samples taken from the 160 kDa peak showed strong and CD7-restricted apoptosis induction towards MOLT-16 cells that was completely abrogated when cells were pretreated for 3 hours with CD7-blocking MAb TH69. Samples taken from the 700 kDa peak showed strong apoptosis induction towards both CD7-positive MOLT-16 cells and CD7-negative Ramos cells, while apoptosis induction could not be inhibited by pre-treatment with MAb TH69. Fractions from the 700 kDa peak were discarded. Fractions from the 160 kDa peak containing homotrimeric scFvCD7:sFASL were pooled and used for further experimental procedures and analyses. Subsequently, we analyzed for the secondary formation of scFvCD7:sFASL multimers or aggregates. This analysis indicated that homotrimeric scFvCD7:sFASL is stable for up to 7 days, with no detectable secondary formation of multimers or aggregates (data not shown). Furthermore, scFvCD7:sFASL incubated for up to 9 days at 37°C in the presence of serum retained potent and CD7-restricted apoptotic activity towards FASL-sensitive MOLT-16 cells (Fig.1B).

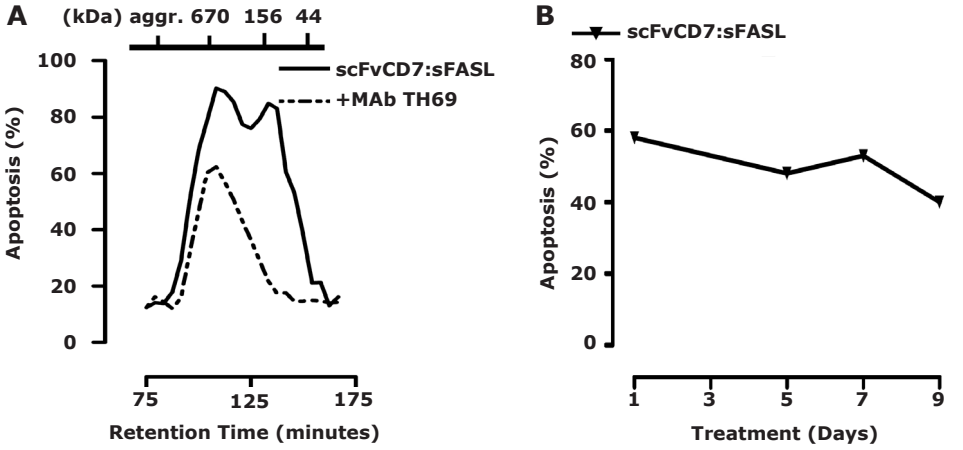

Fig.1. Solution behavior and stability of scFvCD7:sFASL. A; Culture medium derived from CHO-K1 production cells containing scFvCD7:sFASL was subjected to SE-FPLC using a calibrated HiLoad 16/60 FPLC column. The apoptotic activity of each individual fraction was assessed using FASL-sensitive CD7-positive MOLT-16 cells. **B;** ScFvCD7:sFASL was stored for up to 9 days at 37°C in the presence of 10% serum. At serial time-points the remaining apoptotic activity was assessed using FASL-sensitive CD7-positive CEM cells. Apoptosis was assessed by Δψ.

CD7-restricted induction of apoptosis by homotrimeric scFvCD7:sFASL

Incubation of CEM cells with homotrimeric scFvCD7:sFASL resulted in strong and specific binding to the cell surface (Fig.2A, solid line), which was inhibited by pre-incubation with MAb TH69 (Fig.2A, dotted line). Treatment of a series of CD7-positive leukemic T cell lines with serially increasing concentrations of homotrimeric scFvCD7:sFASL (1–110 ng/ml) resulted in potent and dose-dependent increase in apoptosis induction in all CD7-positive T cell lines tested (Fig.2B; Jurkat, 82%; CEM, 90%; HuT-78, 70%; MOLT-16, 98%). CD7-negative FASL-sensitive Raji cells were fully resistant to apoptosis induction even at the highest concentrations tested (Fig.2B; 2.0%). Apoptosis induction by homotrimeric scFvCD7:sFASL was associated with apoptotic DNA-fragmentation that was inhibited by pre-treatment with MAb TH69 (Fig.2C). Similarly, co-incubation with FASL-neutralizing MAb Alf2.1 completely abrogated apoptotic DNA-fragmentation (Fig.2C).

Potent anti-tumour bystander apoptosis induction by homotrimeric scFvCD7:sFASL

Cell surface accretion of homotrimeric scFvCD7:sFASL on CD7-positive target cells can be exploited to crosslink FAS on neighbouring tumour cells that are devoid of CD7-expression, a principle known as the bystander effect[45]. The pro-apoptotic bystander effect of homotrimeric scFvCD7:sFASL was assessed using mixed culture experiments in which Ramos.CD7+ target cells were mixed with CD7-negative Raji bystander cells (ratio

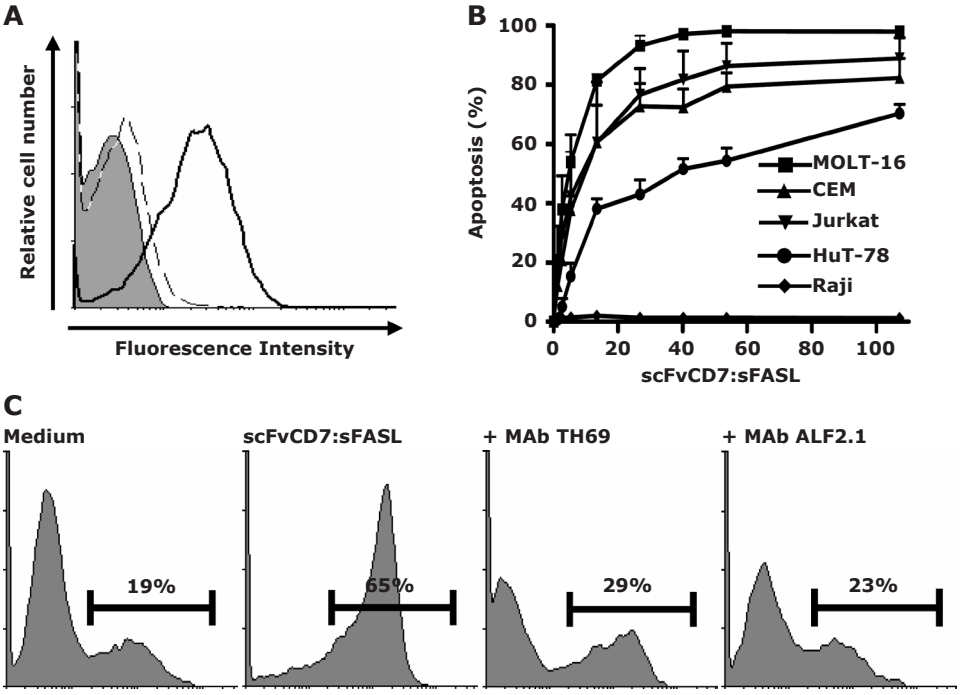

Fig.2. CD7-restricted binding and apoptosis induction by scFvCD7:sFASL A; CD7-restricted binding of scFvCD7:sFASL was assessed using CD7-positive CEM cells. Cells were incubated with scFvCD7:sFASL (solid line) or with unconditioned medium (solid fill). Specific binding was demonstrated by pre-incubating CEM cells with MAb TH69 followed by incubation with scFvCD7:sFASL (dashed line). Binding of scFvCD7:sFASL was assessed by flow cytometry using a PE-conjugated anti-FASL MAb. **B:** CD7-restricted apoptosis induction by scFvCD7:sFASL was assessed using CD7-positive cells (MOLT-16, CEM, Jurkat, HuT-78) and CD7-negative Raji cells. All cell types were treated for 16 h with increasing concentrations of scFvCD7:sFASL, after which apoptosis was assessed by Δψ. Indicated values are mean + standard error of the mean of three independent experiments. **C:** Jurkat cells were treated with scFvCD7:sFASL (20 ng/ml) in the presence or absence of MAb TH69 or FASL-neutralizing MAb Alf2.1. Apoptosis was assessed by apoptotic DNA-fragmentation using MAb F7-26.

1:1). In the Raji bystander cells, a bystander apoptotic effect of up to 31% was detected (Fig.3), which was specifically inhibited in the presence of MAb TH69 (7%) or MAb ALF2.1 (0.2%). No apoptosis induction was observed when parental CD7-negative Ramos cells were used in this experiment (data not shown).

Absence of apoptotic activity of homotrimeric scFvCD7:sFASL towards PBLs and leukocytes

A subpopulation of normal PBLs is CD7-positive and might sustain collateral damage during treatment. Therefore, we investigated the apoptotic activity of homotrimeric

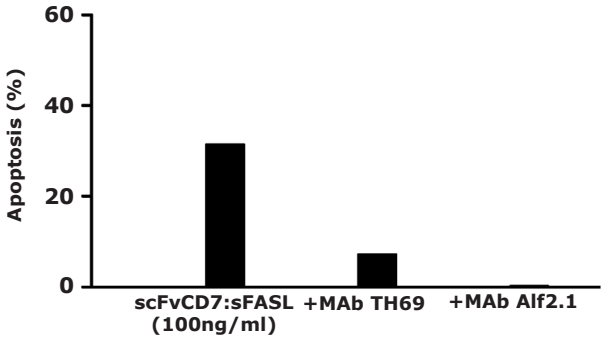

Fig.3. Potent induction of apoptosis in CD7-negative bystander tumor cells. Mixed cultures of Ramos.CD7 target cells and Raji bystander cells (ratio 1:1) were treated for 16 h with scFvCD7:sFASL (100 ng/ml) in the presence or absence of MAb TH69 or MAb Alf2.1. The differential fluorescent labeling of target and bystander cell populations was used to separately evaluate apoptosis induction in the bystander population by Δψ. Indicated values are representatives of three independent experiments.

scFvCD7:sFASL towards normal PBLs. To this end, PBLs were treated with excess amounts of scFvCD7:sFASL (325 ng/ml) for up to 9 days (Fig.4A). This treatment revealed no increase in apoptosis compared to medium control (Fig.4A). Next, we assessed a possible 'innocent' bystander apoptotic effect towards normal human leukocytes when homotrimeric scFvCD7:sFASL is present in a membrane bound state at the cell surface of CD7-positive tumour cells (Jurkat). Treatment of mixed cultures of Jurkat cells and freshly isolated leukocytes (ratio 4:1) with homotrimeric scFvCD7:sFASL revealed no increase in apoptosis in leukocytes (Fig.4B; PBLs, 8%; Jurkat, 72%).

Moderate apoptotic activity of homotrimeric scFvCD7:sFASL towards activated T-cells

Treatment of antiCD3/IL-2 activated T-cells with excess amounts of homotrimeric scFvCD7:sFASL (325 ng/ml) induced apoptosis in approximately 35% of activated T-cells at day 1 (Fig. 4C). Up to day 9, the level of apoptosis in scFvCD7:sFASL treated cells was consistently higher (~10%) than in cells incubated with medium only.

No apoptotic activity towards resting and activated HUVECs

To simulate the effect of scFvCD7:sFASL on human endothelial cells, resting and TNF-α or IFN-γ activated HUVECs were treated with excess amounts of homotrimeric scFvCD7:sFASL. No apoptosis was detected after 24h treatment with homotrimeric scFvCD7:sFASL in either resting - or activated HUVECs (Fig.4D), whereas treatment with excess amount of Actinomycin D resulted in significant induction of apoptosis (53%, 55%, and 56%, respectively).

When homotrimeric scFvCD7:sFASL is bound to the cell surface of circulating leukemic

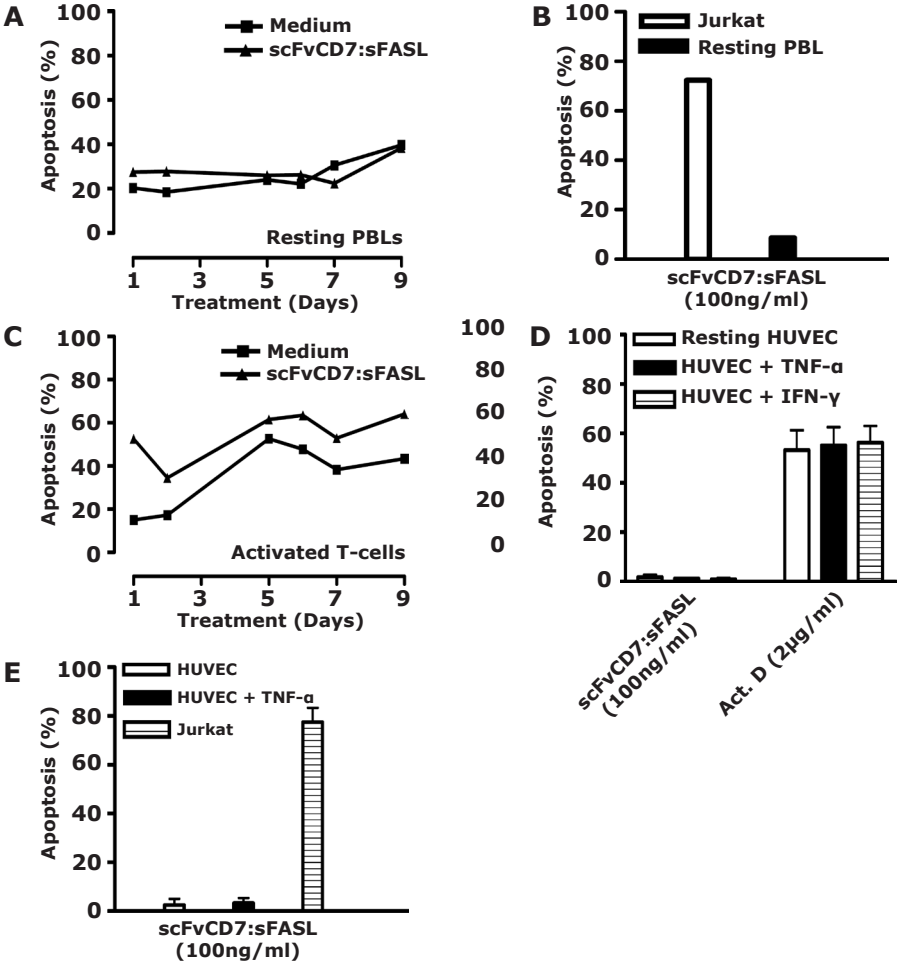

Fig.4. Treatment of normal human leukocytes, activated T-cells and HUVECs with scFvCD7:sFASL. A; Resting PBLs were subjected to treatment with scFvCD7:sFASL (325 ng/ml) or unconditioned medium for up to 9 days, after which apoptosis was assessed by AnnexinV/PI staining. Indicated values are representatives of three independent experiments. **B;** Isolated PBLs were mixed at a ratio of 1:10 with DiI-labeled Jurkat cells. Mixed cultures were treated for 24h with scFvCD7:sFASL (100 ng/ml). Differential fluorescent labeling of Jurkat target cells and PBLs was used to separately evaluate apoptosis induction by AnnexinV staining. Indicated values are representatives of three independent experiments **C;** Activated T-cells were subjected to treatment with scFvCD7:sFASL (325 ng/ml) or unconditioned medium for up to 9 days, after which apoptosis was assessed by AnnexinV/PI staining. Indicated values are representatives of three independent experiments. **D;** Resting, TNF-α or IFN-γ activated HUVEC cells were treated for 24 h with medium, scFvCD7:sFASL (100 ng/ml) or Actinomycin D (2 μg/ml). Apoptosis was assessed by Δψ. **E;** Resting, TNF-α or IFN-γ-activated HUVEC cells were mixed with fluorescently labeled Jurkat cells (ratio 1:1) and treated with scFvCD7:sFASL (100 ng/ml) or Actinomycin D (2 μg/ml) for 24 h. Differential fluorescent labeling of Jurkat target cells and HUVEC bystander cells was used to separately evaluate apoptosis by Δψ.

T-cells there might be a possible innocent bystander effect towards endothelial cells. We simulated this situation using mixed culture experiments in which HUVECs were co-cultured with Jurkat cells (ratio 1:1) in the presence of homotrimeric scFvCD7:sFASL. In this mixed culture experiment, resting and activated HUVECs proved to be fully resistant to a possible innocent bystander effect of homotrimeric scFvCD7:sFASL treatment (Fig.4E; resting HUVEC, 2%; TNF-α activated HUVEC, 3%; Jurkat, 77%).

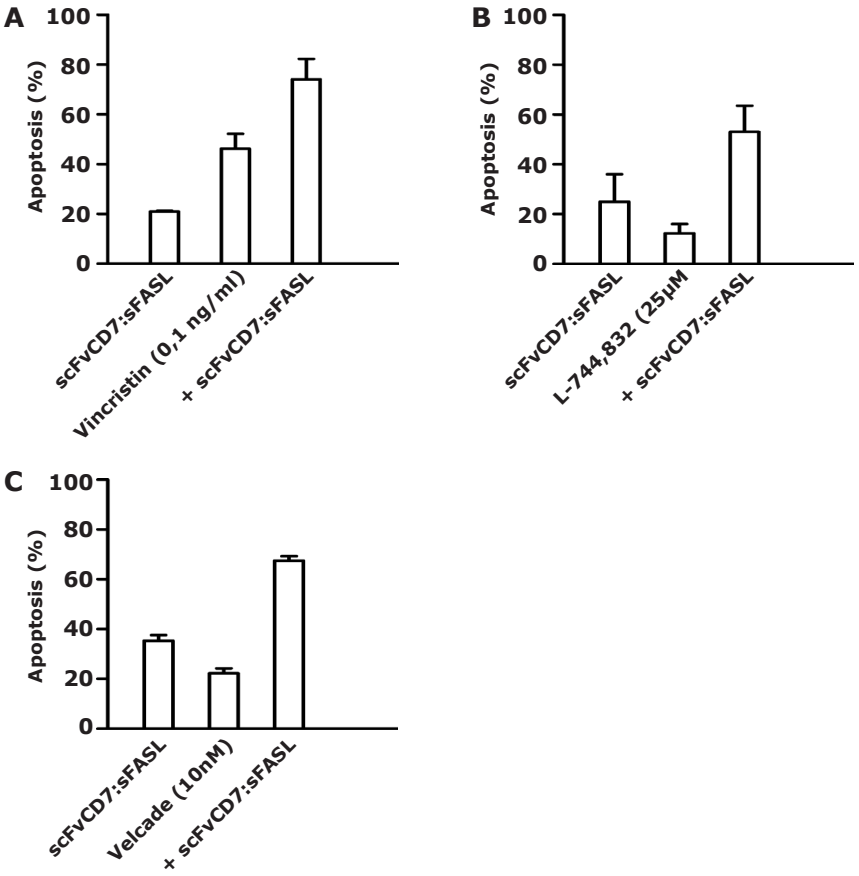

Fig.5. Additive tumoricidal effect of scFvCD7:sFASL with several classes of anti-leukemia agents. Jurkat cells were treated for 16 h either alone or with scFvCD7:sFASL at the indicated concentrations combined with **A;** Vincristin (0.1 ng/ml), **B;** farnesyl transferase inhibitor L-744,832 (25 µM) and **C;** proteasome inhibitor Velcade (10 nM). Apoptosis induction was assessed by Δψ. Synergy was determined using the cooperativity index as described in materials and methods section.

Additive tumoricidal effects of scFvCD7:sFASL with chemotherapeutics and small inhibitory molecules

Sensitivity to FASL-induced apoptosis has previously been shown to be augmented by pre- or co-treatment with various chemotherapeutics and small inhibitory molecules. To establish whether scFvCD7:sFASL had similar properties, Jurkat cells were simultaneously treated with homotrimeric scFvCD7:sFASL, the microtubule inhibitor vincristin, the farnesyl transferase inhibitor L-744,832, or the proteasome inhibitor Velcade. Co-treatment with homotrimeric scFvCD7:sFASL and Vincristin resulted in a synergistic increase in apoptosis compared to either agent alone (Fig.5A; CI=0.9). Co-treatment with L-744,832 or with Velcade similarly increased apoptosis in a synergistic manner (Fig.5B and C, CI=0.7 and 0.85, respectively). Identical treatment of PBLs, activated T-cells or resting and activated HUVECs did not result in a significant increase in apoptosis compared to single agent treatment with the respective chemotherapeutic (Fig.6A, B, and C).

Treatment in vitro of T-ALL, PTCL, and CD7-positive AML patient-derived leukemic blood samples

Blood samples derived from 6 patients suffering from various forms of CD7-positive leukemia were treated *in vitro* with homotrimeric scFvCD7:sFASL. The samples included T-ALL (patients #1 to #4), PTCL (patient #5), and CD7-positive AML (patient #6), all containing >90% leukemic cells (Fig.7A). In three out of four T-ALL patient samples, treatment with homotrimeric scFvCD7:sFASL resulted in a marked increase in apoptosis induction (34%, 56%, and 53% for patients #1,#3, and #4, respectively). Tumour cells from T-ALL patient #2 were refractory to treatment (7% apoptosis). Tumour cells derived from patient #5 (PTCL) showed a moderate response of 14%, while treatment of tumour cells derived from patient #6 (CD7-positive AML) resulted in a 25% increase in apoptosis. Co-incubation with MAb TH69 inhibited apoptosis induction by homotrimeric scFvCD7:sFASL, as exemplified for patient #6 (CD7-positive AML) (Fig.7B).

After treatment, cells from patient #4 were analyzed for the formation of active caspase 3 using MAb 5A1 and evaluated by fluorescence microscopy. Specific staining indicated the activation of caspase 3, whereas untreated cells showed no formation of active caspase 3 (Fig.7C).

Additive apoptotic effect of chemotherapy on primary leukemic cells

Blood samples derived from patient #6 (CD7-positive AML) were treated simultaneously with scFvCD7:sFASL and either vincristin (1 ng/ml) or amsacrine (1 μM) (Fig.7D). In both cases, combination treatment resulted in an additive induction of apoptosis (Fig.7D; vincristin, CI=1.0; amsacrine, CI=1.0).

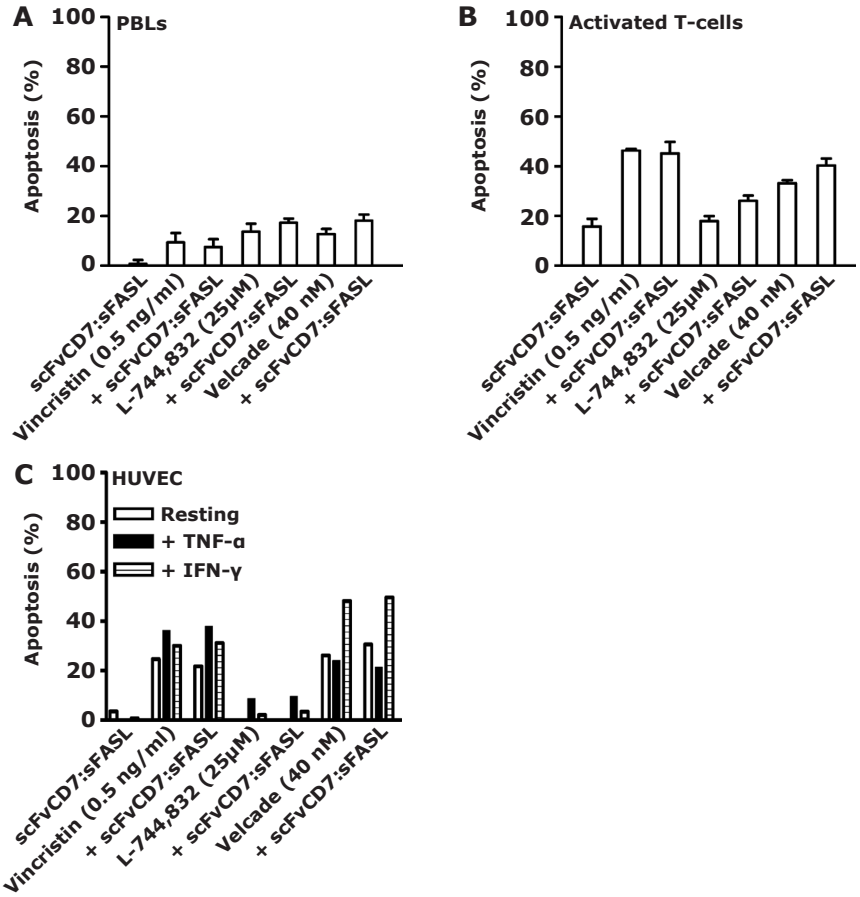

Fig.6. Co-treatment of PBLs, activated T-cells or HUVECs with scFvCD7:sFASL and several classes of anti-leukemia agents. A: PBLs, **B:** activated T-cells and **C:** resting, TNF-α or INF-γ activated HUVECs were treated with scFvCD7:sFASL (40 ng/ml) combined with vincristin (0,5 ng/ml), L-744,832 (25 µM) or Velcade (40 nM). Apoptosis was assessed by AnnexinV/PI staining.

Discussion

Agonistic anti-FAS antibodies and multimeric recombinant FASL preparations show highly potent anti-leukemia activity. However, attempts to exploit these conventional FAS agonists for cancer therapy have met with unacceptable systemic toxicity, largely excluding FAS signalling as a therapeutic strategy for treatment of human malignancies. The toxicity observed for sFASL preparations appears to be directly related to presence

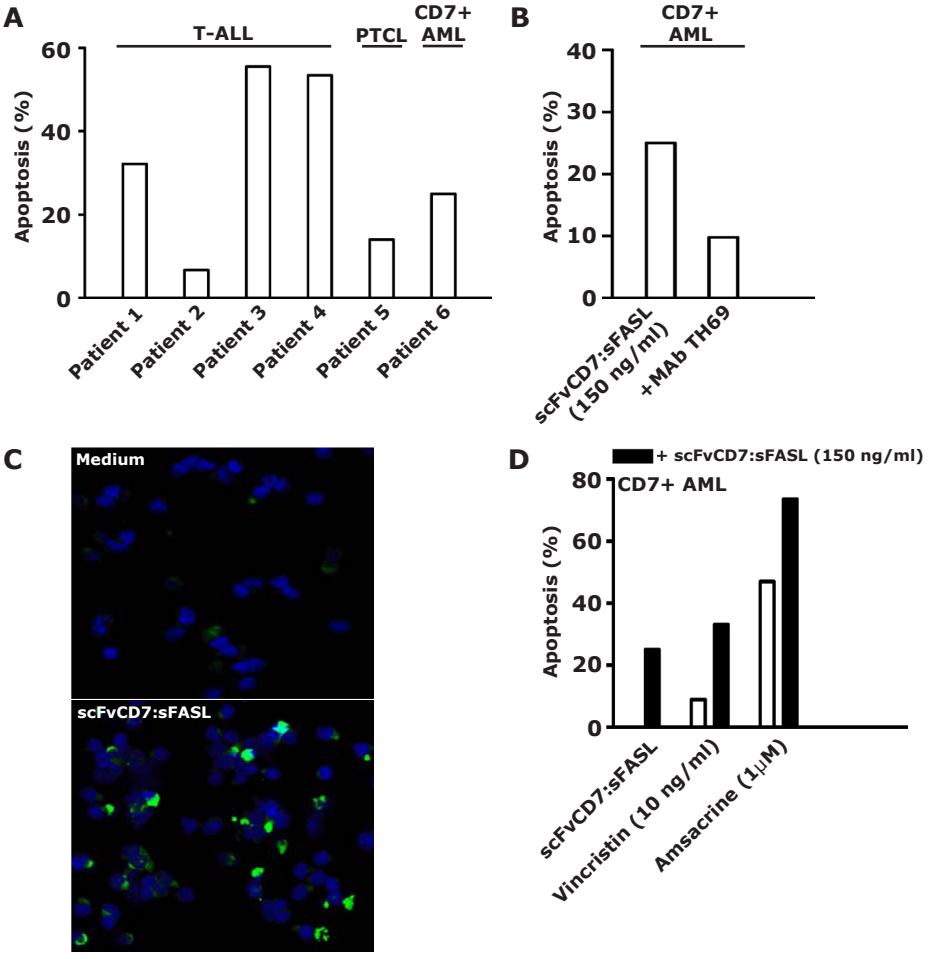

Fig.7. Treatment of T-ALL, PTCL, and CD7-positive AML patient-derived blood samples *in vitro*. A; Blood cells derived from four T-ALL patients, one PTCL patient, and one CD7-positive AML patient were subjected to treatment with scFvCD7:sFASL (150 ng/ml) and analyzed for apoptosis induction using AnnexinV/PI staining. **B;** Primary CD7-positive AML cells were subjected to treatment with scFvCD7:sFASL alone or in the presence of MAb TH69 for 16 h, after which apoptosis was assessed by AnnexinV/PI staining. **C;** Primary T-ALL cells were subjected to scFvCD7:sFASL (150 ng/ml) and then assessed for the presence of active caspase-3 using fluorescent microscopy. **D;** Primary CD7-positive AML cells were subjected to single agent treatment with scFvCD7:sFASL (100 ng/ml), vincristin (10 ng/ml), Amsacrine (1 μM) or to combination treatment. Apoptosis was assessed by AnnexinV/PI staining. Additive induction of apoptosis was determined using the cooperativity index.

of multimeric - and aggregated sFASL species that are frequently formed during or after overexpression in primitive host cells such as *Escherichia coli*. Moreover, it was shown

that as few as two adjacent trimeric (hexameric) sFASL molecules are already sufficient for FAS signalling in Raji, Jurkat, and activated T-cells, as was evidenced by the formation of a death-inducing signalling complex (DISC) and subsequent induction of apoptosis[53]. We produced scFvCD7:sFASL in CHO-K1 cells, a currently favoured industrial host cell type for the production of therapeutic recombinant proteins. We employed a murine kappa light-chain leader peptide to direct the produced fusion protein through the ER and Golgi complex taking advantage of the associated stringent quality control mechanisms that facilitates the secretion of correctly folded and non-aggregated fusion protein into the culture medium. Using SE-chromatography we removed most, if not all, unwanted multimeric forms of scFvCD7:sFASL. Importantly, homotrimeric scFvCD7:sFASL did not display any detectable secondary aggregate formation even after prolonged storage at 37°C in the presence of serum, whereas the apoptotic activity was retained up to this time-point. From this we conclude that scFvCD7:sFASL can be produced as soluble homogeneous homotrimers with no or only minimal secondary aggregate formation.

These features opened the possibility to specifically target biologically inactive homotrimeric scFvCD7sFASL to CD7-positive cells after which sFASL activation is induced by immobilization and multimerization on the target cell surface. Treatment of a number of CD7-positive leukemia T-cell lines with scFvCD7:sFASL showed potent and dose-dependent induction of apoptosis, which was specifically inhibited when cells were pre-treated with MAb TH69 or co-incubated with FAS-neutralizing MAb Alf2.1. Flowcytometric analysis identified that binding of scFvCD7:sFASL to FAS was barely detectable (data not shown). This can be explained by the fact that antibody-based proteins typically have fast-on and slow off-binding rates (for review see[46]), whereas the binding of FASL to FAS (both trimers) is characterized by fast on/fast off rates[47], typical for cytokine/cytokine receptor interaction. Moreover, in homotrimeric scFvCD7:sFASL binding via the antibody domains substantially benefits from the associated avidity effect of the presence of three scFv reading heads[48, 49]. From this it can be concluded that biologically inactive homotrimeric scFvCD7:sFASL acquires full bioactivity only upon binding to cell surface-expressed CD7 and that apoptosis is commenced by reciprocal crosslinking of FAS in an autocrine or paracrine manner.

Using conventional MAb-based therapies target antigen-negative tumour cells can readily escape from therapy[50,51]. The paracrine activation of FAS by CD7-immobilized scFvCD7:sFASL opens the possibility to induce apoptosis in neighbouring FASL-sensitive leukemia cells that, for some reason, have lost CD7 expression. Previously, we reported on an exceptionally strong anti-tumour bystander effect for an analogous scFv:sTRAIL fusion protein[47]. We assessed this mode of action of scFvCD7:sFASL using mixed culture experiments in which CD7-positive target cells were co-cultured with CD7-negative bystander tumour cells. In the bystander tumour cell population a

strong apoptotic effect was detected, which was specifically inhibited in the presence of MAb TH69 or MAb ALF2.1. Taken together, this indicates that the bystander effect of scFvCD7:sFASL predominantly depends on activation of scFvCD7:sFASL on the cell surface of CD7-positive tumour cells. In contrast, only moderate bystander effects have been reported for immunotoxin-based strategies, most likely because these strategies usually depend on a number of consecutive features, including internalization, inter-cellular gap junction communication, and enzymatic conversion[57,58].

Previously, Samel et al provided proof of principle of targeted FAS signalling by fusing sFASL to a scFv specific for the fibroblast activation protein FAP, a tumour-associated stroma marker. The intravenous application of this novel FAS-reagent in mice revealed no signs of systemic toxicity and prevented growth of xenotransplanted FAP-positive, but not FAP-negative, tumour cells[52]. Nevertheless, from these elegant experiments it can not be concluded that in humans a similar favourable toxicity profile will be observed.

In the current study we explored the feasibility and safety of scFv-targeted FAS signalling *in vitro* by treating various CD7-positive human leukemia types in the absence or presence of normal human blood cells and endothelial cells (HUVEC). Treatment of PBLs with scFvCD7:sFASL did not induce apoptosis in any of the normal cell types present, including resting CD7-positive T-cells and CD7-positive NK-cells. This is a remarkable finding since specific binding of scFvCD7:sFASL to CD7 on these cells results in the local activation of scFvCD7:sFASL, that is subsequently able to perform autocrine or paracrine FAS signalling. Apparently, normal cell types are relatively resistant to this form of FAS signalling. In contrast, treatment of antiCD3/IL-2 activated T-cells with homotrimeric scFvCD7:sFASL induced apoptosis in approximately 35% of activated T-cells at day 1 (Fig.4C). Up to day 9, the level of apoptosis in scFvCD7:sFASL treated cells was consistently higher (~10%) than in cells incubated with medium only. It is well established that fratricidal FAS/FASL interactions between activated T-cells are paramount to the effective resolution of an immune response[53]. Although not studied here, we speculate that fusion proteins such as scFvCD7:sFASL, or those that target activation markers such as CD69, can be applied to treat T cell-mediated autoimmunity. We established that resting HUVEC cells are fully resistant to scFvCD7:sFASL treatment. Importantly, also activated HUVEC cells, known to express FAS at the cell surface[51,52], proved to be resistant to homotrimeric scFvCD7:sFASL.

Upon *in vivo* application in leukemia patients many different cell types will simultaneously encounter either free or cell-bound scFvCD7:sFASL. Binding of homotrimeric scFvCD7:sFASL to the cell surface of abundantly circulating leukemic T-cells might lead to a potentially harmful innocent bystander effect towards normal cells e.g. endothelial cells. We simulated this situation using mixed culture experiments in which HUVECs were co-cultured with CD7-positive Jurkat cells (ratio 1:1) in the presence of homotrimeric

scFvCD7:sFASL. In this experiment both resting and activated HUVECs proved to be fully resistant to a possible innocent bystander effect of scFvCD7:sFASL treatment (Fig.4E).
Subsequently, we treated blood samples derived from 6 patients suffering from various forms of CD7-positive leukemia with homotrimeric scFvCD7:sFASL. Samples, all containing >90% leukemic, were derived from four T-ALL patients, one PTCL patient and one CD7-positive AML patient, respectively. Three out of four T-ALL patient samples showed a marked increase in apoptosis induction (32%, 56%, and 53% for patients #1, #3, and #4 respectively). Tumour cells from T-ALL patient #2 (7% apoptosis) were refractory to treatment. Tumour cells derived from patient #5 (PTCL) showed a moderate response of 14%, while treatment of tumour cells derived from patient #6 (CD7-positive AML) resulted in a 25% increase in apoptosis. At first glance, the therapeutic effect of scFvCD7:sFASL towards these primary tumour cells might seem rather moderate. However, *ex vivo* primary tumour cells typically grow in a non-synchronized way at relatively low cell division rates. Previously, it was shown that leukemia cells are only sensitive to FASL-induced apoptosis in the G1 phase of the cell cycle[62,63]. Consequently, the effect observed *ex vivo* may be an underestimation of the therapeutic effect of scFvCD7:sFASL when applied *in vivo*. Moreover, repeated rounds of treatment, as well as treatment with synchronizing agents might help to overcome cell cycle-related resistance.
Various chemotherapeutic agents are known to sensitize tumour cells to FAS-mediated apoptosis at distinct levels, including receptor-proximal, mitochondrial, and/or effector-caspase level. We subjected blood samples derived from patient #6 (CD7-positive AML) to co-treatment with scFvCD7:sFASL and sub-optimal concentrations of vincristin and amsacrine, both chemotherapeutic agents that are already part of clinical practice. Co-treatment resulted in a promising additive effect on apoptosis induction (Fig.7D). Importantly, identical treatment of normal PBLs, activated T-cells, and resting/activated HUVEC did not result in significant induction of apoptosis compared to chemotherapy alone.
In conclusion, we describe a novel and promising anti-T cell leukemia agent that shows strong and CD7-restricted tumoricidal activity towards various CD7-positive leukemic cell types that can be augmented with various chemotherapeutic agents and small inhibitory molecules, such as velcade. Toxicity towards normal cells appears to be restricted to a sub-set of activated T cells. Obviously, more research is needed to evaluate toxicity towards other cells and tissues with an emphasis on liver toxicity. New *in vitro* technologies including the use of human liver organ slices appear to be appropriate for this purpose. Alternatively, scFvCD7:sFASL might be an excellent candidate for the purging of bone marrow from malignant CD7-positive cells. In each case further pre-clinical evaluation for scFvCD7:sFASL is warranted.

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Summary and perspectives

Chapter 8

It has become increasingly clear that aberrancies in the cellular apoptotic machinery play an important role in the process of malignant transformation and tumor progression. Intriguingly, cancer cells are often reliant on these aberrancies for continued survival. Therefore, tumor-selective re-activation of apoptosis represents a promising therapeutic strategy, which in this thesis was pursued by tumor-selective delivery and activation of the Death Inducing Ligands TRAIL and FASL.

Both TRAIL and FASL are essential effector molecules that are active on the cell surface of various immune effector cells. Interestingly, TRAIL and FASL, in particular the soluble derivatives thereof, also possess promising tumor-selective activity in their own right. Therefore, both sTRAIL and sFASL are of considerable interest as anti-cancer agents. However, for their clinical use one can anticipate a number of fundamental problems that need to be addressed first. This includes the differential affinities and crosslinking requirements of sTRAIL for the various TRAIL receptors.

Previously, it has been reported that sTRAIL preferentially binds to TRAIL-R2 over TRAIL-R1 due to the respective affinities of ≤2 nM versus 70nM for these receptors. From these differential affinities it can be predicted that in a therapeutic setting conventional sTRAIL preferentially binds to TRAIL-R2, whereas TRAIL-R2 is actually rather unresponsive to conventional sTRAIL. Consequently, relatively high doses of conventional sTRAIL are needed to reach the therapeutic threshold for TRAIL-R2 signaling at the site of tumor.

In contrast, the specific binding of scFv:sTRAIL fusion proteins to a pre-selected target antigen results in selective accretion at the cell surface of targeted tumor cells. Consequently, the concentration of fusion protein is locally increased and the respective fusion protein is converted into a membrane-bound molecule. As a result, apoptotic signaling by both TRAIL-R1 and -R2 is efficiently activated.

In addition, it has recently been uncovered that different tumor types preferentially signal apoptosis via either TRAIL-R1 or TRAIL-R2. Solid tumors such as colon and breast carcinoma are predominantly sensitive to activation of apoptosis via TRAIL-R2 signaling. In contrast, hematological malignancies, such as B-cell chronic lymphocytic leukemia, are predominantly sensitive to activation of apoptosis via TRAIL-R1 signaling. Several research groups have generated TRAIL-R1 and/or TRAIL-R2 selective mutants of sTRAIL that show enhanced and selective pro-apoptotic activity towards tumor cells expressing the relevant TRAIL-receptor. Therefore, we have exchanged the wild-type sTRAIL domain for either a TRAIL-R1 or a TRAIL-R2 selective mutant in some of our fusion proteins. Research is currently ongoing to determine their potential increase in selectivity and efficacy on selected tumor types.

An important feature of both scFv:sTRAIL and scFv:sFASL fusion proteins is their so-called anti-tumor bystander activity as discussed in **chapters 4** and **7**. The robust anti-

tumor bystander activity of both scFv:sTRAIL and scFv:sFASL fusion proteins may be of clinical relevance to attack target antigen-negative tumor cells that would otherwise escape from targeted therapy.

Obviously, the choice of the targeted antigen is of the utmost importance to maximize the tumor-selective activity of our fusion proteins. Unfortunately, fully tumor-specific cell surface antigens are very rare and perhaps even non-existent or may be unpractical for clinical use. As a second best option, target antigens should be highly (over)expressed in neoplastic tissues and should preferentially play fundamental roles in the pathogenesis of cancer. Ideally, target antigen activation (e.g. phosphorylation) correlates with its function and signaling can be pharmacologically inhibited. Merely binding of the antibody fragment to this target should already result in an anti-tumor effect and/or should sensitize the targeted cell to TRAIL or FASL-mediated apoptosis.
These notions led us to select human EGFR as a highly promising candidate target antigen for scFv:sTRAIL fusion proteins, as described in **chapter 5**. Indeed, targeted delivery of sTRAIL to the EGFR, using EGFR-blocking antibody fragment scFv425, simultaneously inhibited EGFR-mitogenic signaling, sensitized tumor cells to apoptosis, and activated TRAIL receptor apoptotic signaling. Interestingly, co-treatment of EGFR-positive tumor cells with scFv425:sTRAIL and the EGFR-tyrosine kinase inhibitor Iressa synergistically induced apoptosis, indicating the potential clinical benefit of simultaneous extra- and intra-cellular inhibition of EGFR-signaling.
Of particular interest is EGFRvIII, a mutant form of EGFR that is expressed on the cell surface of glioma and in lung and ovarian carcinoma[1-3]. Importantly, EGFRvIII is not detectably expressed by normal tissues, including those tissues with high levels of wild-type EGFR expression. We have constructed fusion proteins containing an scFv with high affinity specificity for EGFRvIII. Unfortunately, production of the anti-EGFRvIII:sTRAIL fusion protein in CHO cells yielded high-levels of aggregated fusion protein, unsuitable for EGFRvIII-restricted induction of apoptosis (unpublished data). We are currently trying to resolve this technical problem that is most likely related to improper protein folding of this fusion protein during protein synthesis.
Taken together, when compared to conventional sTRAIL and sFASL both scFv:sTRAIL and scFv:sFASL fusion proteins have strongly improved therapeutic characteristics. The choice for therapeutic application of either molecule may depend on the characteristics and requirements of the particular disease to be treated. These characteristics and requirements need to be investigated in greater detail in order to further tailor and optimize the respective fusion protein for a particular disease.

The pre-clinical data in this thesis provide clear proof of principle for the therapeutic potential

of targeted delivery and local activation of sTRAIL and sFASL for cancer therapy. Obviously, several aspects still need to be addressed in order to determine the clinical feasibility of this approach. An essential step is to determine the safety and efficacy of scFv:sTRAIL and scFv:sFASL fusion proteins in relevant animal models. In this respect, *in vivo* studies are currently being conducted in our lab in which human cell lines are xenografted into nude mice. The cell lines used have been lentivirally equipped with the luciferase gene to allow for *in vivo* real-time monitoring of tumor growth and response to therapy using bioluminescent imaging[4]. Recently, novel fluorescent probes have been developed for real-time *in vivo* imaging of caspase-8 and caspase-3 activation[5]. These probes appear to be ideal to accurately image and measure therapeutic apoptosis induction during treatment with our fusion proteins. Simultaneously, these probes can also be used to monitor the development of unwanted collateral apoptosis induction in normal cells and tissues.
Additionally, the tissue distribution and half-life of the respective scFv:sTRAIL/ scFv:sFASL fusion proteins needs to be accurately assessed.

Alternative target antigens and targeting domains
As indicated above the choice of the particular target antigen for our fusion proteins may be of eminent importance for tumor cell-restricted delivery and efficient activation of apoptosis. This is exemplified by the specific inhibition of the mitotic signaling activity of EGFR by scFv425:sTRAIL, as delineated in **chapter 5**. This strategy can be extended to other target antigens that are involved in complementary or alternative apoptotic signaling routes. An interesting example of such a target antigen is CD20, which upon cross-linking by the monoclonal antibody Rituximab potently activates apoptosis[6]. Our preliminary experiments indicate that scFvRit:sFASL, a fusion protein comprising a Rituximab-derived scFv antibody fragment genetically fused to sFASL, simultaneously activates CD20- and FAS-apoptotic signaling pathways. The preliminary data indicate that scFvRit:sFASL shows superior apoptotic activity compared to the parental Rituximab antibody.

Sometimes it may be necessary or even beneficial to deliver sTRAIL and sFASL using targeting domains other then those derived from antibodies. This is for instance the case when the encoding cDNAs for the VH and VL domains of a given anti-tumor antibody are not (yet) available or obtainable.
In addition, when the target antigen of interest is a receptor of some sort, it may be useful to deliver sTRAIL or sFASL by exploiting the receptor's natural ligand. An interesting example that we are currently working on is the recently identified ligand of CD7, designated K12[7]. We selected K12 because of its low nanomolar affinity for the CD7 antigen. Furthermore, it is known that CD7 is critically involved in activation of apoptosis

after cross-linking by another potential CD7 ligand, the lectin Galectin-1. We anticipate that our K12:sFASL and K12:sTRAIL fusion proteins display enhanced targeting and pro-apoptotic activity towards CD7-positive tumor cells.

Mammalian antibodies and (recombinant) derivatives thereof have long been the paradigm for targeted delivery of therapeutics. Recently, several alternative and artificial targeting domains coming from (non-)mammalian species have been identified. Repeat proteins such as ankyrin repeat (AR), leucine-rich repeat (LRR), or tetratricopeptide repeat (TPR) proteins are abundant specific binding molecules in nature. They are composed of small structurally homologous units (repeats) that stack to form a repeat domain. These non-globular repeat proteins have been subjected to protein engineering to serve as binding molecules with strongly improved thermostability[8]. Combinatorial libraries of designed ankyrin repeat proteins (DARPins) have been constructed from which candidate (e.g. tumor-selective) DARPins can be selected at unparalleled speed[9]. Importantly, DARPins can be cheaply produced at very high yields in simple prokaryotic organisms.

Overcoming apoptosis resistance

Literature reports that ~50% of tumor cell lines are resistant to TRAIL. Moreover, TRAIL-resistant cells often show cross-resistance to e.g. chemotherapeutics. The concept outlined here for the targeted delivery of sTRAIL and sFASL will obviously fail when the targeted tumor cells are resistant to apoptosis due to one or more defects in Death Receptor-mediated apoptotic signaling. Such resistance can be due to down-regulation or mutational inactivation of initiator caspase-8 or alternatively by epigenetic silencing of agonistic TRAIL-receptors.

To overcome Death Receptor-related resistance issues, the targeted delivery and activation of other effector moieties that possess divergent pro-apoptotic activity is warranted. Of particular interest are those proteins that play an important role in the elimination of aberrant cells by the immune system. In analogy to TRAIL, the intrinsic selectivity of such physiological effector molecules for diseased or aberrant cells can be exploited.

Worth mentioning in this respect are members of the protein family of Galectins[10]. Galectins are highly conserved animal lectins with beta-galactoside-binding activity for glycosylated proteins. Galectins play key roles in innate and adaptive immune responses through sugar-dependent and -independent mechanisms. The first identified member, Galectin-1, is expressed in a number of different tumor types and was shown to modulate the tumor immune response by eliminating infiltrating T cells. Reversely, several T-ALL leukemia cell lines are sensitive to apoptosis induction by recombinant Galectin-1. Very recently it was shown that multidrug resistant tumor cells of various origins are sensitive to apoptosis induction by Galectin-1.

Of note, for most activities the physiologically active form of Galectin-1 is a homodimer. Unfortunately, the *in vivo* efficacy of Galectin-1 is limited because at lower concentrations the equilibrium is rapidly shifted towards the inactive monomeric form.
However, this apparently unfavorable feature of Galectin-1 may well be exploited by constructing scFv:Galectin-1 fusion proteins. We hypothesize that for scFv:Galectin-1 sufficient tumor cell accretion will be feasible, whereby the therapeutic apoptotic activity of Galectin-1 will be locally (re)activated.

Concluding remarks
An important 'universal' issue in oncology is the available therapeutic window for selective activation of apoptosis in cancer cells. Although the approach outlined in this thesis shows marked selectivity for cancer cells, single agent therapy might prove not selective enough. Seemingly apoptosis-resistant tumor cells may have highly elevated apoptotic thresholds that can only be lowered by a combinatorial use of various pro-apoptotic agents as discussed in **chapter 2**. Some of the prominent examples are agents that are able to re-activate p53 and those that inhibit up-regulated anti-apoptotic proteins such as BCL-2 and XIAP.
Integration of the various anti-cancer concepts may help to rationally design combinatorial treatment strategies that enhance or restore the sensitivity of tumor cells to apoptosis induction. The most promising combinations will probably involve those drugs that work along different or complementary apoptotic signaling routes with non-overlapping toxicities towards normal cells.
The clinical success of such combinatorial strategies will rely heavily on patient-tailored identification of the respective cancer-related aberrancies. This will require the development of reliable, cost-effective and high-throughput diagnostic tools.
Laser-capture microscopy and DNA-micro array technology make it possible to evaluate large quantities of gene-expression data from individual cancer cells. However, currently it is still difficult to extract meaningful information from these data and to relate them to tumor-specific phenomena. Nevertheless, further improvements in this field are anticipated that might help to identify hitherto unknown routes and mediators of tumor-specific apoptosis induction. In turn, these findings might help to identify new targets for cancer cell-restricted activation of apoptosis.

Finally, the field of glycobiology, an as yet under-investigated area, is starting to yield a wealth of information that can be exploited for specific targeting of cancer cells. Glycosylation is a highly diverse and non-template driven process that can generate enormous informational content. Consequently, glycosylation represents one of the most diverse families of recognition patterns. It has since long been recognized that

cancer cells display aberrant glycosylation. Because of the highly specific interactions of these aberrantly glycosylated proteins with physiological receptors, such as members of the Galectin family, cancer specific glycosylation is a promising target for intervention. Indeed, it can be anticipated that as more insight is gained into the glycobiology of cancer, a plethora of specific targets, targeting moieties, and effector moieties will become available for future therapy.

Taken together, as the molecular aberrations in apoptosis regulation in cancer cells are elucidated, the rational design of combinatorial approaches paves the way towards enhanced and tumor-selective apoptosis induction that will help fight cancer in a clinical setting.

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Nederlandse samenvatting

Chapter 9

In het lichaam van een gezond en volwassen individu is het aantal lichaamscellen gemiddeld gesproken constant. Het is van essentieel belang voor het volgroeide lichaam dat er een nauwkeurige balans bestaat tussen de afbraak van oude, beschadigde, of "zieke" cellen en de aanmaak van nieuwe gezonde cellen. Grondslag voor deze balans is een cellulair proces dat apoptose genoemd wordt. Apoptose is een vorm van geprogrammeerde celdood waarbij lichaamscellen zelf en/of onderling zorgen voor een correcte en tijdige vernietiging. Tijdige vernietiging van lichaamcellen, c.q. beperking van hun levensduur, is belangrijk omdat iedere delende cel na verloop van tijd mutaties oploopt in het genetische materiaal, hetgeen uiteindelijk kan leiden tot ongewenst en gevaarlijk cellulair gedrag. Ook in het embryonale stadium is apoptose belangrijk en wel voor het verwijderen van ongewenste cellen en overbodig geworden weefselstructuren.

Apoptose is een nauwkeurig gecontroleerd cellulair proces dat biochemisch gesproken energie kost. Dit in tegenstelling tot ongecontroleerde celdood, ook wel necrose genoemd, waarbij de cel opzwelt en daarna openbarst (cytolyse). Necrose is vaak schadelijk voor het organisme aangezien de hierbij vrijkomen celinhoud omliggende cellen kan aantasten. Bovendien trekt de vrijgekomen celinhoud opruimende cellen van het afweersysteem aan, waarbij een ontstekingsproces op gang komt dat de omliggende weefsels kan beschadigen.
Bij apoptose krimpt een cel, wordt de kern van de cel gefragmenteerd, en valt de cel ten slotte uit elkaar in vele kleine delen (zogeheten apoptotic bodies) zonder dat daarbij de celinhoud vrijkomt. De apoptotische cel en/of de apoptotic bodies worden snel en zonder verdere schade door cellen van het afweersysteem opgeruimd. Om de correcte activatie en executie van apoptose te garanderen beschikt het lichaam over een uitgebreid pro- en anti-apoptotisch regulatiesysteem, waarbij vele verschillende receptoren en signaalverwerkende moleculen nauwkeurig samenwerken.

Onderzoek naar apoptose heeft de laatste decennia een enorme vlucht genomen, wat heeft geleid tot het ophelderen van belangrijke achtergronden van uiteenlopende ziekten. Te veel, te weinig, te late, of afwezigheid van apoptose leidt onherroepelijk tot ziekte. Tussen degeneratieve ziekten van de hersenen, chronische ontstekingen, en kanker lijken op het eerste gezicht weinig overeenkomsten te bestaan, maar elk van deze ziekten wordt gekarakteriseerd door een verstoord apoptotisch proces.
Bij kanker is in het algemeen sprake van een verminderde gevoeligheid voor apoptose. Dit begint reeds bij het ontstaan van kanker. Bepaalde defecten in apoptose kunnen bijvoorbeeld leiden tot een gevaarlijk verlengde levensduur van de cellen, die uiteindelijk resulteert in cellulaire onsterfelijkheid met als gevolg tumorvorming. Aanvankelijk zijn deze tumoren nog goedaardig, maar doordat de tumor op den duur steeds meer

mutaties oploopt, ontstaat na verloop van tijd kwaadaardig cellulair gedrag. Sommige defecten maken kankercellen minder gevoelig voor pro-apoptotische signalen vanuit de extracellulaire omgeving, zoals bijv. afkomstig van buurcellen of cellen van het afweersysteem. Daarnaast kunnen defecten zorgen voor een sterk verhoogde activatie van anti-apoptotische systemen. Tezamen leidt dit er uiteindelijk toe dat kankercellen veel sneller kunnen groeien dan hun normale tegenhangers. Deze versnelde celgroei vergt uiteraard een verhoogd metabolisme in de kankercel en gaat gepaard met het ontstaan van intracellulaire stress. Dergelijke stress maakt cellen, ook kankercellen, gevoeliger voor apoptose. Deze toegenomen gevoeligheid wordt in kankercellen gecompenseerd door een verhoogde activiteit van de anti-apoptotische systemen. In feite staan kankercellen dus klaar om in apoptose te gaan, wat alleen voorkomen wordt door de verhoogde activiteit van anti-apoptotische systemen. Conventionele kankerbehandeling met chemotherapie en bestraling brengt zoveel schade aan in snel groeiende kankercellen dat deze alsnog tot apoptose worden gedwongen. Dit leidt echter ook tot ongewenste schade aan gezonde cellen met alle bekende nadelige gevolgen van dien. De balans in kankercellen tussen pro- en anti-apoptotische systemen selectief beïnvloeden is dan ook de belangrijkste uitdaging voor nieuwe kankertherapieën.

Reeds vele jaren is er onderzoek gedaan naar nieuwe therapieën die specifieker kankercellen kunnen opsporen om ze vervolgens te vernietigen met zo weinig mogelijk schade aan normale cellen. Gezamenlijk worden deze therapieën aangeduid met de Engelse term Targeted Therapy. In zijn algemeenheid wordt hierbij geprobeerd een groeiremmende of hoog toxische stof selectief af te leveren (of werkzaam te laten zijn) op de plek van de tumor. Hierbij wordt vaak gebruik gemaakt van kankerselectieve antilichamen met daaraan gekoppeld een toxische stof (immuuntoxines).
Het antilichaam deel in een immuuntoxine herkent en bindt aan een target eiwit dat voornamelijk (maar niet uitsluitend) voorkomt op het celmembraan van kankercellen. In reactie hierop neemt de kankercel het gehele immuuntoxine op, waarna het toxine gedeelte zorgt voor de antikankeractiviteit. Sommige immuuntoxines laten daarbij veelbelovende selectiviteit en antikankeractiviteit zien. Helaas treden hierbij ook frequent ernstige bijwerkingen op bij normale cellen. Een probleem bij deze en vele andere benaderingen is namelijk dat de moleculen waarop een dergelijke therapie gericht is niet daadwerkelijk kankerspecifiek zijn. Authentiek kankerspecifieke target moleculen zijn voor de meeste vormen van kanker nog niet ontdekt of komen voor in onbruikbaar lage hoeveelheden. Bovendien wordt in de bovengenoemde therapieën gebruik gemaakt van toxines of chemotherapeutica die niet alleen uitermate toxisch zijn voor de kankercellen maar ook voor de normale cellen. Een bijkomende nadelige eigenschap van immuuntoxines is dat zij eerst actief in de cel moeten worden opgenomen om een effect te kunnen hebben,

terwijl een dergelijke opname in bepaalde gevallen juist verstoord is in kankercellen.

In dit promotieonderzoek is een volledig nieuwe benadering gekozen. Deze nieuwe strategie is er op gericht bepaalde apoptoseinducerende eiwitten af te leveren aan het celoppervlak van kankercellen. Met het oog op een aantal bijzondere en veelbelovende eigenschappen is hierbij gekozen voor twee apoptose inducerende eiwitten, TRAIL en FASL, die beide van nature voorkomen op cellen van het menselijke afweersysteem, zoals T-cellen en NK-cellen. Op deze cellen spelen TRAIL en FASL een belangrijke rol bij het selectief activeren van apoptose in onder meer virus geïnfecteerde cellen en kankercellen.
In bepaalde gevallen kan het extracellulaire deel van deze eiwitten van het celoppervlak afgeknipt worden, resulterend in oplosbare sTRAIL en sFASL varianten. Eerder is al aangetoond dat sTRAIL selectief apoptose kan induceren in allerlei typen kankercellen zonder daarbij schade te veroorzaken in normale gezonde cellen. Hoe deze uitzonderlijke intrinsieke selectiviteit voor een kankercel tot stand komt is nog steeds niet volledig opgehelderd. Ook voor sFASL is kankerselectieve apoptose inductie beschreven, maar er zijn ook berichten, dat geaggregeerd (ofwel samengeklonterd) sFASL zeer toxisch is voor normale levercellen. Hiermee raakte sFASL als potentieel geneesmiddel op de achtergrond. Zoals echter later zal worden besproken heeft ons onderzoek, beschreven in **hoofdstuk 7**, aangetoond dat er wel degelijk goede mogelijkheden zijn om sFASL geschikt te maken voor therapeutische toepassingen bij bepaalde vormen van kanker.

Verschillende onderzoeksgroepen en bedrijven over de gehele wereld zijn bezig om sTRAIL geschikt en beschikbaar te maken voor klinische toepassingen. Ondanks de veelbelovende antikankeractiviteit van sTRAIL kleven aan het gebruik ervan in patiënten een aantal voorspelbare fundamentele problemen. Voor TRAIL zijn tenminste 4 verschillende TRAIL-receptoren gevonden waarvan er twee (TRAIL-R1 en TRAIL-R2) daadwerkelijk een apoptose signaal kunnen afgeven aan de kankercel. Al deze verschillende TRAIL receptoren komen voor op vrijwel alle (normale) cellen in het menselijke lichaam. Hierdoor zal bij toediening van sTRAIL het overgrote deel van het toegediende eiwit kunnen binden aan de enorme overmaat van TRAIL-receptoren op gezonde cellen. De binding van sTRAIL aan deze normale cellen hoeft op zich niet per se schadelijk te zijn, maar het vermindert wel in zeer sterke mate de hoeveelheid sTRAIL die nog zal kunnen binden op de plek van de tumor. Wij voorspellen dan ook dat grote en wellicht onhanteerbare hoeveelheden sTRAIL zullen moeten worden toegediend om een therapeutisch effect te bewerkstelligen. Het is bovendien niet uit te sluiten dat bij dergelijke hoeveelheden toch sprake zal zijn van toxische effecten op gezonde cellen.
Een ander probleem is gelegen in het feit dat TRAIL-R1 en TRAIL-R2 verschillend

reageren op correct geproduceerd (trimeer) sTRAIL. TRAIL-R1 geeft een apoptotisch signaal af wanneer sTRAIL bindt aan de receptor. TRAIL-R2 daarentegen geeft alleen een efficiënt apoptotisch signaal af na binding van sTRAIL dat niet correct geproduceert is (multimeer). Tumoren die preferentieel TRAIL-R2 tot expressie brengen blijken derhalve relatief ongevoelig te zijn voor de huidige klinisch relevante sTRAIL preparaten.

Het doel van het onderzoek beschreven in dit proefschrift is het ontwikkelen van een strategie die de antikankeractiviteit van sTRAIL (en zoals later zal worden behandeld van sFASL) sterk verbetert. De door ons gevolgde strategie bestaat uit het genetisch koppelen van sTRAIL aan een kankerselectief antilichaam fragment (scFv). Het aldus geconstrueerde scFv:sTRAIL fusie-eiwit werd ontworpen om met sterk verhoogde selectiviteit en kracht te binden aan kankercellen, om vervolgens alleen lokaal, dus op de plaats van de kankercel, apoptose te activeren. Dit doel wordt onder andere bereikt door de bijzondere bindingseigenschappen van antilichamen. Antilichamen binden razend snel en specifiek aan het relevante target antigen. Eenmaal gebonden zal een antilichaam zeer lang gebonden blijven en soms pas na uren weer vrij komen van het target antigen. Binding van sTRAIL aan TRAIL-receptoren geschiedt ook zeer snel, maar anders dan bij een antilichaam valt het sTRAIL molecuul ook weer zeer snel van zijn receptor af. Op grond hiervan kan worden voorspeld dat conventionele sTRAIL preparaten niet zelfstandig kunnen ophopen op de plaats van de tumor. Dankzij het antilichaam component zal de sTRAIL component zoals aanwezig in het scFv:sTRAIL fusie-eiwit echter wel selectief gebonden blijven aan kankercellen en aldaar veel langer en dus vaker de TRAIL receptoren activeren.

Op grond van het bovenstaande voorspelden wij een aantal bijzondere eigenschappen van scFv:sTRAIL fusie eiwitten.

- Door te binden aan het target antigen op een geïsoleerd gelegen kankercel kan een scFv:sTRAIL fusie-eiwit de op de zelfde cel gelegen TRAIL-receptoren activeren tot apoptose (cellulaire zelfmoord of suïcide).
- Binding van scFv:sTRAIL aan het target antigen kan tevens leiden tot meercellige en wederzijdse interacties waarbij het fusie-eiwit als het ware een dodelijke brug vormt tussen kankercellen onderling. M.a.w. behandeling met scFv:sTRAIL leidt tot reciproque en versterkte activatie van het apoptose programma tussen twee of meer kankercellen (broedermoord ofwel fratricide).
- Bovendien kan de binding van scFv:sTRAIL aan het target antigen leiden tot meercellige interacties, waarbij het fusie-eiwit een dodelijke brug vormt met

kankercellen die zelf geen target antigen op het celoppervlak hebben. Dit effect wordt ook wel bystander effect genoemd.

Het een en ander is schematisch weergegeven in Fig.1 in **hoofdstuk 4**.

Het resultaat van onze aanpak is uiteraard afhankelijk van de keuze van het target antigen waaraan het scFv:sTRAIL fusie-eiwit selectief afgeleverd zal worden. Zoals hierboven reeds werd genoemd, zijn authentiek kankerspecifieke target antigenen zeer zeldzaam of niet beschikbaar. Een goed alternatief is dan een target antigen dat sterk verhoogd op kankercellen voorkomt en relatief weinig op normale cellen. In de laatste decennia is er een veelvoud van dit soort target antigenen geïdentificeerd dat mogelijk gebruikt kan worden voor onze strategie.

Om het werkingsmechanisme van onze nieuwe strategie te onderzoeken hebben we eerst een target antigen gekozen dat zelf geen directe rol speelt in het apoptose proces. De keuze viel daarbij op de Epithelial Glycoprotein-2 (EGP2), ook wel EpCAM (Epithelial Cell Adhesion Molecule) genoemd. EGP2 komt sterk tot expressie op het celoppervlak van de meest frequente menselijke kankersoorten, zoals de epitheliale kankersoorten borstkanker en darmkanker, terwijl normale epitheliale cellen een veel lagere EGP2 expressie hebben. Vervolgens werd het eerste prototype fusie-eiwit geconstrueerd, genaamd scFvC54:sTRAIL, met daarin een antilichaam fragment dat specifiek kan binden aan EGP2.
Onderzoek naar het werkingsmechanisme van scFvC54:sTRAIL wordt in **hoofdstuk 3** beschreven. Uit dit onderzoek kwam duidelijk naar voren dat scFvC54:sTRAIL geheel volgens plan functioneerde. Behandeling van EGP2-positieve kankercellen leidde tot een sterke binding van scFvC54:sTRAIL aan het celoppervlak. Door de selectieve binding aan EGP2 werd het fusie-eiwit omgezet in een membraan gebonden variant van TRAIL, waardoor de TRAIL component zeer efficiënt zowel TRAIL-R1 als TRAIL-R2 activeerde. Dit alles resulteerde in versterkte en selectieve apoptose in kankercellen.

Vervolgens werd gekozen voor een target antigen dat juist wel een duidelijke integrale rol speelt in de gevoeligheid van kankercellen voor apoptose. Als klinisch relevant voorbeeld hiervan werd gekozen voor de EGFR (Epidermal Growth Factor Receptor). Verstoorde EGFR signalering is een vaak voorkomend fenomeen in solide tumoren en stimuleert celgroei, onder andere door actief de resistentie tegen apoptose te verhogen.
Inhibitie van EGFR signalering, door middel van een blokkerend monoklonaal antilichaam (b.v. Cetuximab) of een inhibitor van het intracellulaire signalerende domein van de EGFR (b.v. Iressa), remt kankergroei en herstelt bovendien gedeeltelijk de gevoeligheid voor

apoptose. Deze strategie is al in de kliniek onderzocht, voor zowel Cetuximab als Iressa, wat tot beperkt klinisch succes leidde.
Op basis hiervan hebben wij een scFv:sTRAIL fusie-eiwit ontworpen met specificiteit voor EGFR. In **hoofdstuk 5** zijn de resultaten van het onderzoek naar de werking van dit molecuul weergegeven. Behandeling van EGFR-positieve kankercellen met scFv425:sTRAIL resulteerde in specifieke binding van scFv425:sTRAIL aan de EGFR. Binding leidde bovendien tegelijkertijd tot een snelle remming van EGFR signalering met als gevolg dat de cel gevoeliger werd voor apoptose. Vervolgens activeerde het sTRAIL domein van het fusie-eiwit efficiënt apoptose. Het combineren van scFv425:sTRAIL met Iressa resulteerde bovendien in een nog verder versterkte apoptotische activiteit. Samenvattend, kan scFv425:sTRAIL van grote waarde zijn voor de behandeling van EGFR-positieve kanker soorten.

In de afgelopen jaren is er veel ervaring opgedaan in de kliniek met het toepassen van therapieën gebaseerd op antilichamen. Dat heeft geleid tot de identificatie van enkele problemen. Eén van de grote belemmeringen van antilichaam therapie is het feit dat kankercellen, die het antigen verliezen waartegen de therapie gericht is, niet meer herkend worden. Dergelijke kankercellen kunnen al tijdens de start van de therapie aanwezig zijn, maar kunnen zich ook tijdens de therapie ontwikkelen.
Door de bijzondere eigenschappen kan een scFv:sTRAIL fusie-eiwit ook een sterke apoptotische activiteit bezitten tegen kankercellen die het target antigen verloren hebben. In dit geval zorgt scFv:sTRAIL er namelijk voor, dat target antigen-positieve kankercellen de nabijgelegen target antigen-negatieve kankercellen tot apoptose dwingen; het zogeheten bystander effect.
Onderzoek naar de aard en efficiëntie van dit 'bystander effect' van scFv:sTRAIL is in **hoofdstuk 4** beschreven voor het EGP2-specifieke fusie-eiwit scFvC54:sTRAIL. Binding van scFvC54:sTRAIL aan EGP2 resulteerde in een zeer sterke activatie van apoptose in nabijgelegen EGP2-negatieve cellen. Het bystander effect was specifiek voor kankercellen en trad niet op tegen 'innocent' bystander cellen zoals normale gezonde bloedcellen. Enige voorwaarde voor dit antikanker 'bystander effect' is, dat de cellen functionele TRAIL-receptoren bezitten. Samenvattend kan dit bystander effect van scFv:sTRAIL fusie-eiwitten mogelijk van belang zijn om recidieven te voorkomen die kunnen ontstaan door verlies of heterogene expressie van het target antigen.

Deze preklinische data geven aan dat selectieve aflevering van sTRAIL naar kankercellen een veelbelovende therapeutische strategie kan zijn voor de behandeling van solide tumoren. De compactheid van de tumormassa in deze typen kanker kan echter de penetratie van scFv:sTRAIL fusie-eiwitten belemmeren. De toekomstige klinische

toepassing van scFv:sTRAIL fusie-eiwitten bij solide tumoren is dan ook waarschijnlijk gelegen in de behandeling van patiënten na cytoreductieve therapie, c.q. de behandeling van minimal residual disease.

Voor de toepassing van scFv:sTRAIL fusie-eiwitten voor de behandeling van leukemie (bloedkanker) zal deze problematiek van veel minder/geen invloed zijn, omdat dit type kanker gekenmerkt wordt door een diffuse groei. In het volgende deel van het proefschrift is dan ook onderzoek gedaan naar de toepasbaarheid en de effectiviteit van het selectief afleveren van sTRAIL naar leukemiecellen.
Leukemie wordt gekenmerkt door een kwaadaardige overproductie van witte bloedcellen. Afhankelijk van de soort witte bloedcel die de ziekte veroorzaakt, wordt er gesproken van lymfatische of myeloïde leukemie. De algemene therapie bij leukemie is intensieve chemo- en radiotherapie, eventueel gevolgd door beenmergtransplantatie. Helaas sterven nog ieder jaar betrekkelijk veel mensen, zowel volwassenen als kinderen, aan leukemie door de vaak zware bijverschijnselen en het optreden van resistentie.

Het ontwikkelen van verbeterde therapieën die selectief leukemiecellen elimineren is dus zeer gewenst. In **hoofdstuk 6** is onderzoek beschreven naar de haalbaarheid en effectiviteit van het toepassen van scFv:sTRAIL bij T-cel leukemie. T-cel leukemie is een van de typen leukemie waarbij de huidige behandelingsmethoden maar een zeer beperkt therapeutisch effect hebben en gepaard gaan met een significante morbiditeit. Derhalve is een scFv:sTRAIL fusie-eiwit ontworpen dat specifiek target naar het membraaneiwit CD7, een antigen dat vaak en hoog tot expressie komt op verschillende typen T-cel leukemie. Expressie van CD7 op gezonde cellen is gelimiteerd tot een subset van bloedcellen.
Het door ons ontworpen fusie-eiwit, scFvCD7:sTRAIL, bond selectief aan CD7, resulterend in een sterke activatie van apoptose in T-cel leukemiecellijnen. Belangrijker nog is het feit dat behandeling van leukemiecellen die direct geïsoleerd waren uit het bloed van patiënten, leidde tot veelbelovende apoptotische activiteit. In een rechtstreekse vergelijking met een CD7-specifiek immuuntoxine bleek het scFvCD7:sTRAIL fusie eiwit bovendien een sterkere antileukemische activiteit te bezitten. Dit terwijl de immuuntoxine, in tegenstelling tot scFvCD7:sTRAIL, een hoge ongewenste activiteit tegen normale CD7-positieve bloedcellen bezat. Daarnaast werd ontdekt dat de behandeling van cellen met scFvCD7:sTRAIL in combinatie met verschillende standaard chemotherapeutica resulteerde in een sterk verhoogde apoptotische activiteit. Hierdoor zullen bij combinatie met scFvCD7:sTRAIL de benodigde doses van dit soort chemotherapeutica verlaagd kunnen worden om eenzelfde therapeutisch effect te bereiken. Onze data tonen aan dat het toepassen van scFv:sTRAIL fusie-eiwitten voor de behandeling van leukemie een goede aanvulling kan zijn op bestaande conventionele therapieën.

In vergelijking met sTRAIL heeft het eerder vermelde eiwit sFASL een hogere intrinsieke apoptotische activiteit tegen kankercellen. Daartegenover staat dat sFASL in initiële experimenten, ernstige levertoxiciteit veroorzaakte in muizen. Recent is gebleken dat deze toxiciteit eigenlijk volledig te wijten is aan de incorrecte productie van sFASL, waardoor er eiwitaggregaten ontstaan. Onderzoek met recombinant sFASL heeft bovendien uitgewezen dat de antikankeractiviteit van sFASL pas geactiveerd wordt na vorming van grotere complexen op de kanker cel. Niet gecomplexeerd (trimeer) sFASL bleek in feite geen biologische activiteit te bezitten tegen kankercellen. Dit in principe veelbelovende eiwit kan dus alleen succesvol worden toegepast als sFASL uitsluitend op de plek van de tumor kan worden geactiveerd.

Op basis van deze gegevens voorspelden wij dat het genetisch koppelen van sFASL aan een tumorspecifiek scFv antilichaam fragment zou resulteren in een dergelijke lokale activatie van sFASL. Derhalve werd door ons een scFv:sFASL fusie-eiwit ontworpen. Het antilichaam fragment van een dergelijk scFv:sFASL fusie eiwit zal snel en specifiek aan het relevante target antigen binden en, zodra gebonden, ook langdurig gebonden blijven. De sFASL component van het scFv:sFASL fusie-eiwit zal dankzij deze binding van het antilichaam fragment lokaal omgezet worden in een membraangebonden FASL. Hierdoor wordt scFv:sFASL lokaal actief, waarna apoptose geïnduceerd wordt in de leukemiecel via de receptor FAS.

Het onderzoek naar de effectiviteit van een dergelijk scFv:sFASL fusie-eiwit gericht tegen het antigen CD7 wordt in **hoofdstuk 7** beschreven. Uit dit onderzoek bleek duidelijk dat dit scFvCD7:sFASL fusie-eiwit lokaal een sterke antileukemische activiteit bezat. Selectieve binding van scFvCD7:sFASL aan CD7 resulteerde in een sterke activering van apoptose in CD7-positieve leukemische cellijnen. Van groot belang is dat scFvCD7:sFASL ook sterk apoptose induceerde in leukemische cellen die geïsoleerd waren uit patiëntenbloed. De activiteit van scFvCD7:sFASL werd bovendien in belangrijke mate versterkt door het fusie-eiwit te gebruiken in combinatie met verschillende conventionele chemotherapeutica en andere nieuwe experimentele middelen.
Door het selectief afleveren van sFASL naar CD7 bezit dit fusie eiwit ook een sterk anti-leukemisch 'bystander' effect tegen leukemiecellen die CD7-negatief zijn. Tegen normale gezonde cellen is dit fusie eiwit grotendeels inactief; alleen geactiveerde T cellen bleken enigszins gevoelig te zijn voor scFvCD7:sFASL. Dit is echter in lijn met de verwachting, aangezien FASL normaal een belangrijke rol speelt bij het elimineren van geactiveerde T-cellen tijdens de afschakeling van een immuunrespons. Samenvattend vormen de preklinische data beschreven in dit hoofdstuk een sterke aanwijzing dat de lokale

activering van sFASL door middel van scFvCD7:sFASL een veelbelovende therapeutische strategie kan zijn voor de behandeling van CD7-positieve leukemiën.

In **hoofdstuk 8** wordt een korte samenvatting gegeven van de resultaten die beschreven zijn in de **hoofdstukken 3** tot en met **7**, waarbij tegelijkertijd de perspectieven voor de verdere ontwikkeling van deze therapeutische strategie besproken worden.

Dankwoord

chapter 10

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